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ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DPN	Diphosphopyridine nucleotide
EDTA	Ethylenediaminetetra-acetate
FMN	Flavin mononucleotide
FAD	Flavin–adenine dinucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
TPN	Triphosphopyridine nucleotide
tris	2-Amino-2-hydroxymethylpropane-1:3-diol

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Enzymic Desulphation of Porphyran

By D. A. REES*
Department of Chemistry, University College of North Wales, Bangor, Caernarvonshire

(*Received* 2 *January* 1961)

In a previous paper (Peat & Rees, 1961) it was noted that galactose seems to occupy an important position in the carbohydrate metabolism of the red seaweeds. The results of a survey of some of the enzymes present in extracts of a representative of this class (*Porphyra umbilicalis*) were described and discussed, particular attention being paid to the enzymic transformations of the two important galactose-containing carbohydrate components of the alga: floridoside (glycerol 2-α-D-galactopyranoside) and porphyran. The latter substance is a complex sulphated polysaccharide containing residues of D-galactose, L-galactose, 6-*O*-methyl-D-galactose and 3:6-anhydro-L-galactose (Peat, Turvey & Rees, 1961). This paper describes the partial purification, assay and some of the properties of the enzyme, present in *Porphyra* extracts, that catalyses the release of sulphate from the ester linkages of porphyran. In the earlier paper it was assumed that this reaction proceeds by the usual hydrolytic mechanism, and the enzyme was therefore named porphyran sulphatase. Recent work [for a preliminary account, see Rees (1961)] has revealed that this assumption may not be correct; in the present paper the question of nomenclature will be left open, and the enzyme catalysing sulphate release will be referred to as 'the enzyme'.

EXPERIMENTAL

Reagents. All chemicals used in the activation–inhibition experiments, and for the preparation of buffer solutions, were AnalaR reagents, except zinc acetate, which was 'laboratory reagent' grade (British Drug Houses Ltd.).

Viscometric detection of enzyme activity. This was carried out as described by Peat & Rees (1961).

Enzyme incubations and assay of sulphate-liberating enzyme. The assay medium usually contained porphyran (1%, w/v, in water; 1 ml.) together with purified freeze-dried enzyme (0·05–0·1 mg.) and the specified electrolyte solution (0·5 ml.). When working with buffer solutions other than borate, it was often necessary to use up to 0·2 mg. of enzyme protein. The incubation period was normally 90 min. at 35°. The reaction was stopped by adding trichloroacetic acid (aqueous 40%, w/v, solution; 0·1 ml.) with shaking, and the protein coagulated by warming on a water bath at 85°. The solution was clarified on the centrifuge, and a portion (1·0 ml.) withdrawn into a dry tube and brought to 25° in a thermostat. Barium chloride solution [containing 67 g. of barium chloride dihydrate and 32 ml. of concentrated hydrochloric acid (sp.gr. 1·18)/l.; 2·0 ml.], which had been prewarmed in the same thermostat, was added rapidly with shaking, and the mixture left at 25° for 5 min. At the end of this time the extinction of the barium sulphate suspension was measured at 500 mμ against a water blank in the Unicam SP. 500 spectrophotometer, with 1 cm. glass cells. Control determinations were carried out by adding trichloroacetic acid to similar digests immediately on mixing and then proceeding as described above. A typical calibration curve is shown in Fig. 1. Factors affecting the accuracy and reproducibility of the method are discussed below. The activity of the enzyme was expressed as μg. of sulphate liberated under these conditions. Neither the enzyme nor porphyran liberated free sulphate when incubated alone.

Choice of conditions for the extraction of the enzyme. As a preliminary to this work, it was desirable to find extraction conditions giving a high yield of enzyme. To this end, portions of fresh or frozen seaweed (100 g.) were minced into a variety of solvents (150 ml.) at 4°. After standing for 24 hr. at the same temperature, the solutions were filtered through muslin, clarified on the centrifuge and dialysed against running tap water at 5° in the presence of toluene until free of sulphate, the dialysis sac being changed daily. The resulting solutions were then assayed for enzyme.

Preparation of partially purified enzyme. The entire operation was carried out in the cold room at 0–4°. The following is a typical experiment. Fresh, pressed seaweed (3 kg.; Peat & Rees, 1961), collected in the period February to June 1960, was washed with tap water and minced (Kenwood mixer) into aqueous sodium carbonate (0·25%; 4·5 l.) which had been precooled to 4°. After 24 hr., the solution (pH 8·3) was squeezed through muslin, adjusted with dilute acetic acid to pH 6·0–6·5 and clarified on the centrifuge (1000–2000*g*). The amount of free sulphate found to be present in the solution by the assay method described above was about 180 μg./ml. A pilot experiment was next performed to ascertain how much adsorbent was required to remove the enzyme from solution. To each of a series of tubes containing samples of the seaweed extract (2 ml.) was added a different amount of calcium phosphate gel suspension [prepared according to Keilin & Hartree (1938) and washed thoroughly with water before use, since the enzyme is inhibited by both calcium and phosphate; containing 24 mg. dry wt./ml.; 0·03–1·0 ml.]. The solutions were well mixed and left for 20 min., with intermittent shaking. The calcium phosphate was separated on the centrifuge and a portion of the supernatant (0·5 ml.) incubated with the polysaccharide (1%, w/v; 1 ml.) and buffer (0·1 M-sodium tetraborate–hydrochloric acid, pH 7·6; 0·10 ml.) overnight for assay. The results could only be regarded as semiquantitative, because the measured extinctions were beyond the

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calibrated range, due to the rather high initial concentration of free sulphate. Nevertheless, it was clear that approximately 90% of the activity was removed by the addition of 0·17 ml. of gel suspension. To the remainder of the seaweed extract, therefore, the necessary quantity of suspension was added to remove this amount of enzyme (85 ml. of gel/l. of extract). After 30 min. with intermittent stirring, the gel was collected on the centrifuge. Preliminary experiments had indicated that acetate buffer is a suitable solvent for eluting the activity. The gel was washed twice with portions (800 ml. each) of 0·1 M-sodium acetate (pH 6·5) by shaking with glass beads in the presence of a few drops of octan-2-ol for 15 min., the washings, which contained negligible activity, being rejected. The enzyme was eluted by treating the gel in the same way with 1 M-sodium acetate (pH 7·5; 7 × 500 ml.), the calcium phosphate being recovered on the centrifuge between treatments. The combined eluates were adjusted to 0·8 saturation with solid ammonium sulphate, and the precipitate was isolated on the centrifuge and dissolved in water (100–200 ml.). This solution was dialysed against running water at 2° until little sulphate remained, and freeze-dried to a pink powder (0·12 g.).

RESULTS

Turbidimetric assay of enzyme

Accuracy of the method. When the procedure for the estimation of sulphate was standardized with respect to the factors discussed below, it was found to give results to within ±5% of the expected value. A typical calibration curve is shown in Fig. 1. This does not pass through the origin since the deproteinized digests were slightly coloured.

Age of the barium chloride solution. It has long been recognized that the age of the barium chloride solution used in turbidimetric methods for the estimation of sulphate influences the turbidity of the resultant barium sulphate suspension (Snell & Snell, 1948). For this reason, many workers have preferred to use barium chloride crystals, or have specified fresh barium chloride solution. In the present work it was found that freshly prepared barium chloride solution did in fact give a suspension with a higher extinction than did a solution which was some days old. This effect was observed consistently, but since the difference was only one of 5–7% it was ignored when the purpose of the experiment was to compare the sulphate released in digests run simultaneously. When accuracy was required in an absolute sense, the reagent was, of course, recalibrated. The barium chloride reagent was discarded when it began to give erratic results.

Time allowed to elapse after mixing. The extinction of the barium sulphate suspension varies markedly with the time allowed to elapse after mixing. Fig. 2 shows this variation, and from this it is clear that the reading must be taken 5–20 min. after mixing the solutions.

Influence of added materials. In the experiments described in this paper, the overall concentration of buffer in the assay medium was usually less than 0·05 M. It was found that the same calibration curve could be used when working with phosphate, acetate, borate and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers in this concentration range. The reagents listed in Table 2 were similarly without effect when they were present at the indicated concentrations. Assay mixtures containing the same amount of free sulphate, but widely different amounts of protein or carbohydrates, gave readings differing by up to about 25%. However, variations in the amount of protein present of several-fold at low concentrations did not markedly alter the results, and it was only when investigating the effect of substrate concentration on the reaction over a wide range that it was necessary to introduce corrections in the present study.

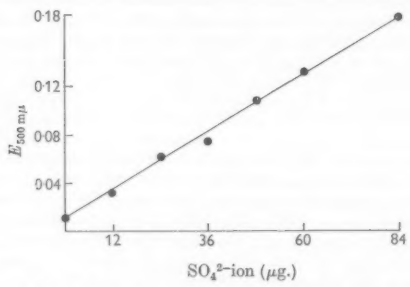

Fig. 1. Typical calibration curve for sulphate estimation. Solutions were prepared by mixing crude enzyme solution (Peat & Rees, 1961; 0·5%, 0·7 ml.) with sodium tetraborate–hydrochloric acid buffer (0·1 M, 0·15 ml.) and standard sulphate solution (0·7 ml.). These were deproteinized and estimated as described in the Experimental section. Each point is the mean of two readings.

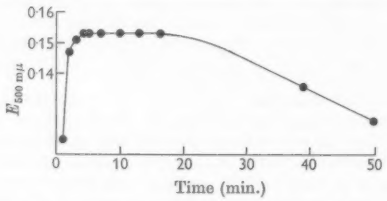

Fig. 2. Variation with time of extinction of a typical barium sulphate suspension. Porphyran (1%, w/v, solution, 1·0 ml.) and purified enzyme (about 0·1 mg. in 0·5 ml. of 0·03 M-sodium tetraborate–hydrochloric acid buffer, pH 7·6) were mixed and incubated at 35° for 1 hr. The digest was deproteinized and treated as described in the Experimental section to obtain a barium sulphate suspension. This was transferred to a glass spectrophotometer cell and extinction measured at intervals.

Extraction and purification of the enzyme

Choice of conditions for the extraction. The results of the brief survey are given in Table 1. It is clear that sodium carbonate is the most effective solvent of those studied, and that it is preferable to mince the seaweed in the fresh rather than the frozen state.

Purification of the enzyme. Adsorption on calcium phosphate followed by elution with sodium acetate proved to be a satisfactory method of purification. The extent of the purification achieved was estimated by comparing the amounts of crude and purified preparations which exhibited the same activity. Similar results were obtained whether the comparison was made in terms of the dry weight after dialysis and freeze-drying, or in terms of E at 280 mμ as a measure of protein concentration. In a typical experiment the purification was 22-fold, with a recovery of 53% of the original activity.

Stability of the product. The enzyme gradually lost its activity when stored in solution at 4°, but it could be stored for at least several months at room temperature as a freeze-dried powder.

Properties of the partially purified enzyme

Presence of another enzyme with action on porphyran. It has been shown that the unfractionated seaweed extract contains a depolymerase which decreases the viscosity of porphyran (Peat & Rees, 1961). The purified enzyme still showed this activity.

Activity of the product. About 0·10 mg. of the freeze-dried preparation, when incubated with 10 mg. of porphyran in an overall volume of 1·5 ml. of 0·01 M-sodium tetraborate–hydrochloric acid buffer, at pH 7·6 and 35°, released 80 μg. of free sulphate in the first hour.

Table 1. *Comparison of the sulphate-liberating activities of* Porphyra *extracts prepared under various conditions*

State of specimen	Solvent	Sulphate liberated in 3 hr. from assay mixture* (μg.)
Fresh	Water	20
	0·1 M-Sodium acetate, pH 7·5	24
	1·0 M-Sodium acetate, pH 7·5	26
	0·25% Sodium carbonate	30
Frozen	Water	10
	0·1 M-Sodium acetate, pH 7·5	15
	1·0 M-Sodium acetate, pH 7·5	17
	0·25% Sodium carbonate	17

* Contains 0·70 ml. of porphyran solution (1%, w/v), 0·70 ml. of extract (see text) and 0·15 ml. of 0·10 M-sodium tetraborate–hydrochloric acid buffer, pH 6·5.

Table 2. *Activation and inhibition of the enzyme*

Digests were prepared as indicated in the Experimental section, with an overall buffer concentration (acetate, pH 7·6) of 0·05 M, except when the effect of phosphate was being studied, when the only electrolyte added was the indicated concentration of sodium phosphate buffer, pH 7·6. The control digests contained only acetate buffer.

Reagent	Concentration (mM)	Sulphate liberated (% of control)
KCN	5	0
NH_4F	50	50
Cd^{2+}	7	0
Cu^{2+}	7	0
Al^{3+}	7	0
Ni^{2+}	7	15
Ca^{2+}	100	0
K^+	3	140*
Mn^{2+}	3	140*
	30	20
Zn^{2+}	3	140*
	30	20
Co^{2+}	3	140*
	30	0
Mg^{2+}	3	140*
	30	25
Ethylenediaminetetra-acetic acid	0·3	10
	3	0
Phosphate	10	70†
Borate		(see text)

* These figures are variable, depending on the metal-cation content of the enzyme preparation.

† The enzyme used in this experiment was eluted from calcium phosphate by 0·2 M-phosphate but not 0·01 M-phosphate, the procedure being otherwise similar to that described.

Activation and inhibition by reagents other than borate. The effect on the enzyme of adding a number of reagents is shown in Table 2. Complete inhibition was achieved by the addition of metal-binding reagents, thus indicating that there is a bi- or ter-valent cation essential to the enzyme. No inhibition was observed when a mixture of Zn^{2+} or Co^{2+} ions (1·5 mM) and ethylenediaminetetra-acetic acid (EDTA) (0·5 mM) was present. There was 50% inhibition when Mn^{2+} ion was substituted for one of these cations, and complete inhibition when Mg^{2+} ion was used.

Activation by borate. The most powerful activator was borate, which produced 60% activation of the enzyme at pH 7·6. The addition of Mg^{2+} or K^+ ions to the assay medium containing borate produced further activation, the total activation being the sum of the activations produced separately by borate and by the appropriate cation. The curves of enzyme activity against buffer concentration showed an increase to a maximum value,

after which the activity diminished (Fig. 3). The buffer concentration at which maximum activity occurred varied with pH (Fig. 3), thus suggesting that the species responsible for inhibition is one which is present in greater concentration at the more alkaline pH.

Optimum pH *and substrate concentration.* Because of the enhancement of activity in borate buffer of suitable concentration, this medium was adopted for routine use. The optimum pH was in the region 7·6–7·8 in both 0·01 M-sodium tetraborate–hydrochloric acid (Fig. 4) and tris–hydrochloric acid buffers. The enzyme was approximately saturated with respect to substrate when the latter was present in 0·7 % solution.

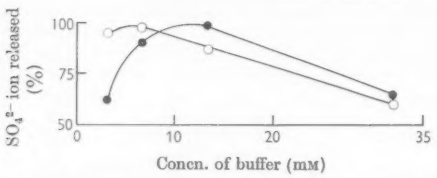

Fig. 3. Variation of enzyme activity with concentration of sodium tetraborate–hydrochloric acid buffer: ●, at pH 6·5; ○, at pH 7·6. Values for sulphate released are expressed as percentages of the maximum observed value at the same pH. Digests were prepared, incubated and assayed as described in the Experimental section.

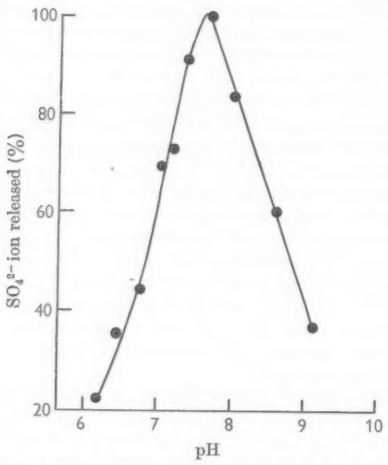

Fig. 4. Variation of enzyme activity with pH in 0·01 M-sodium tetraborate–hydrochloric acid buffer. Values for sulphate released are expressed as percentages of the maximum observed value (pH 7·7). The digests were prepared, incubated and assayed as indicated in the Experimental section.

DISCUSSION

Turbidimetric and nephelometric methods have long been used for the determination of inorganic sulphate (Snell & Snell, 1948), and the assay method for sulphate-liberating enzyme reported here is an adaptation of this well-established principle. Previous methods have been modified because of the special problems posed by the nature of the assay mixture, and in order to gain sensitivity. The present method is rapid and reproducible provided that care is taken to standardize the conditions under which the barium sulphate suspension is prepared and measured. The most widely used method for the assay of carbohydrate sulphatases has been the benzidine method of Dodgson & Spencer (1953), and, although the present method is both less sensitive and less accurate than this (± 5 % as compared with ± 2 %), it is more rapid and its precision was judged sufficient for this work. It should be noted, however, that the relatively poor sensitivity of the method made it necessary to use rather long incubation periods. For this reason, when the amount of enzyme present was plotted against the amount of sulphate released, the curve was slightly convex to the sulphate axis. It is therefore probable that for more precise studies it would be advisable to revert to the benzidine method, when short incubation periods could be used. A similar point has been made by Dodgson (1961). Since this manuscript was first submitted, another variation of the turbidimetric method for sulphatase assay has been described (Dodgson, 1961), but this is stated to be unsuitable for use with polysaccharide substrates. The present method does not suffer from this limitation, and is therefore regarded as complementary to that of Dodgson.

It emerges from the activation–inhibition experiments that the enzyme is very much affected by the ions present in the incubation mixture. The enzyme is evidently dependent on the presence of a bi- or ter-valent metal ion for its activity, because it is completely inhibited in the presence of metal-binding agents. The addition of Mg^{2+} ions to the EDTA-inactivated enzyme failed to restore the activity, and therefore Mg^{2+} ion neither satisfies the requirements of the enzyme nor liberates the essential cation from its EDTA complex. The essential cation is therefore not Mg^{2+}, but one for which EDTA has greater affinity. In this respect the enzyme can be contrasted with many enzymes involved in the biological transformations of phosphate esters, for which Mg^{2+} ion is an essential cofactor. Further experiments directed towards the identification of the essential cation, and the category of metal-dependent enzymes to which the sulphate-liberating enzyme belongs,

have been hampered by the fact that the loss of activity in the presence of EDTA rapidly becomes irreversible on standing. It is inadvisable to draw further conclusions on the basis of the available evidence, since the enzyme is still in a highly impure state. It is possible that the observed inhibition by cyanide (Table 2) is due to cyanide binding of the essential cation, although other mechanisms are possible (Dixon & Webb, 1958).

Since porphyran is itself a polyelectrolyte, it is conceivable that certain of the activators influence the reaction by interaction with the substrate rather than the enzyme. It is known that salts present in solution with polysaccharide polyanions can alter the configuration of the polymer, and it is possible that some of the cation activators (Table 2) operate by increasing the time spent by the substrate in a configuration favourable for reaction. The observed activation by borate might be induced by an analogous mechanism, the borate forming a complex with the hydroxyl groups of the polymer, thus altering the molecular charge and configuration.

SUMMARY

1. The enzyme responsible for the desulphation of porphyran by extracts of *Porphyra umbilicalis* has been purified 22-fold by adsorption on calcium phosphate gel followed by elution with sodium acetate.

2. A turbidimetric assay method has been developed.

3. The effect on the enzyme of pH and of various activators and inhibitors has been studied. It is dependent on the presence of a bi- or ter-valent cation, which is not Mg^{2+}, and is markedly activated by borate.

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Loss of Liver Glycogen after Administration of Protein or Amino Acids

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In a previous report (Clark, Goodlad, Chisholm & Munro, 1960), it was shown that the feeding of protein can cause rapid changes in the amount of adenosine triphosphate in the liver. Rats were given a carbohydrate-rich meal in order to produce a high concentration of adenosine triphosphate in the liver over a period of several hours. When casein was then fed to such animals, the adenosine triphosphate concentration fell rapidly. Further investigations showed that this fall was accompanied by a considerable decrease in the glycogen content of the liver.

In the present paper, we have explored the changes in the glycogen content of the liver caused by protein administration. It has been shown that there is also a rapid disappearance of glycogen from the liver after feeding several amino acids individually. Along with the decrease in glycogen content, there is a fall in the total carbohydrate content of the liver and a decrease in the blood-sugar concentration, but no change in muscle-glycogen concentration. Loss of liver glycogen occurs after administration of protein or amino acids to adrenodemedullated rats or to alloxan-diabetic rats. The nature of the mechanism underlying this action of dietary protein is discussed.

EXPERIMENTAL

Animals. Each experiment was carried out on a group of female albino rats of closely similar weight. In different experiments mean weight varied from 125 to 180 g. The animals were housed in individual cages.

Feeding procedure. On the day of killing, the rats were fed in the morning with a carbohydrate-rich meal, which

caused deposition of glycogen in their livers; some hours later, a meal of protein or a single amino acid was given and the effect of this on liver-glycogen concentration was measured. In order to train the rats to consume these meals promptly, they were fed for about a week beforehand on a synthetic diet given as two meals (Goodlad & Munro, 1959). At 9 a.m. they received 5 g. of carbohydrate together with vitamins and minerals, and at 5 p.m. they ate 4·2 g. of a protein–carbohydrate–fat mixture. On the final day, they received the carbohydrate-rich meal in the morning as usual, but the evening meal at 5 p.m. was replaced by the test protein or amino acid fed alone, and the animals were killed at various times up to 2 hr. later. Control rats, which received no food at 5 p.m., were killed at the same time-intervals and the glycogen content of the livers of fed and control groups were compared.

The casein used as the test protein was commercially purchased (unextracted grade, Glaxo Ltd., Greenford, Middlesex). The amino acids fed were obtained from British Drug Houses Ltd., Poole, Dorset. Glutamic acid was neutralized with $NaHCO_3$ before it was given.

Analytical procedures. When only the glycogen concentration in liver was studied, the rats were killed by a blow on the head and the livers were excised immediately. When blood-sugar and muscle-glycogen concentrations were also measured, the animals were first anaesthetized with Nembutal. Blood was then withdrawn by syringe from the inferior vena cava and specimens of muscle were obtained from the fronts of both thighs.

The samples of liver and muscle (about 2·5 g.) were rapidly weighed and transferred to 6 ml. of hot 30% (w/v) KOH. After heating in a water bath at 100° for 15 min., glycogen was precipitated by addition of 1·2 vol. of absolute ethanol (Good, Kramer & Somogyi, 1933). After standing overnight at room temperature, the precipitated glycogen was separated by centrifuging and dissolved in water. The amount of glycogen was then estimated by the anthrone procedure as described by Trevelyan & Harrison (1952), and the results were expressed as g. of glycogen/100 g. wet weight of liver.

The rapidity of transfer of the liver and muscle specimens to KOH was found to influence their glycogen content. Removal of both tissues, weighing and immersion in hot KOH could be accomplished within 1 min. If the liver and muscle specimens were kept for a further 4 min. before being plunged into KOH, their glycogen contents were 11 and 19% lower respectively (mean of 3 experiments). From these findings it was concluded that important losses of glycogen were unlikely to occur during the minute needed for rapid removal of liver and muscle specimens.

In one experiment, the total carbohydrate content of the liver was estimated in place of glycogen. Specimens of liver (4 g.) were dissolved in 12 ml. of hot 30% KOH and made up to 50 ml. with water. Estimations of total carbohydrate were carried out by the anthrone procedure; the presence of protein in the dissolved liver preparation does not seriously affect the anthrone determination (Seifter, Dayton, Novic & Muntwyler, 1950).

The reducing-sugar content of the blood specimens taken from the inferior vena cava was estimated by the Hagedorn & Jensen (1923) procedure and in a few instances by glucose oxidase (Huggett & Nixon, 1957), with reagents supplied by C. F. Boehringer und Soehne GmbH., Mannheim, Germany.

Adrenodemedullation. This was carried out by the technique of Evans (1936). The cortex was allowed to regenerate for 1–2 months before commencing the dietary experiment. The completeness of removal of the medulla was confirmed by histological examination at the time of killing. Animals not completely demedullated were rejected.

Alloxan-diabetic rats. Diabetes were produced by subcutaneous injection of 200 mg. of alloxan/kg. body wt. The alloxan was dissolved in citrate–phosphate buffer, pH 4·0, in order to stabilize it (Klebanoff & Greenbaum, 1954). The occurrence of diabetes was recognized by the appearance of glucose in the urine obtained 3 days after injection, and was confirmed by measurements of blood-sugar concentration at the time of killing, which was generally the tenth day after injection with alloxan; only animals with blood-sugar concentrations above 200 mg./100 ml. were accepted as diabetic.

RESULTS

A number of workers (e.g. Barnes, 1953; Andrews, 1960) have commented on the difficulty of obtaining reproducible concentrations of glycogen in the livers of mice and rats. In the present series of experiments this variability was reduced by using rats that had been trained to consume food promptly. On the day of the experiment, they were fed a meal of carbohydrate which was eaten immediately and they were killed 9–10 hr. later. When no intervening food was given, this resulted in a liver-glycogen concentration of 2–3% at the time of death.

Changes in liver-glycogen concentration produced by feeding different amounts of casein. When rats prepared in this way are fed casein 2 hr. before being killed, there is a sharp decrease in the amount of glycogen found in the liver at death (Clark *et al.* 1960). Table 1 shows that the extent of this effect is related to the amount of casein fed. When 2·5 g. of casein was given, the glycogen concentration fell to one-tenth of that in the livers of the control

Table 1. *Changes in liver-glycogen concentration produced by feeding different amounts of casein to rats*

A standard carbohydrate-rich meal was fed at 9 a.m. At 5 p.m. the rats received the amounts of casein indicated below, and were killed 2 hr. later. Each entry is the mean (±S.E.) of observations on three animals.

Amount of casein fed (g.)	Mean body wt. (g.)	Liver-glycogen concn. (g./100 g. of liver)
Nil	144	2·36±0·42
0·5	147	1·15±0·11*
1·0	142	0·84±0·31*
2·5	142	0·20±0·03†

* Value differs significantly from the control value ($P < 0.05$ by t test).

† Value differs significantly from the control value ($P < 0.01$ by t test).

Table 2. *Effect of feeding single amino acids or olive oil on liver-glycogen concentration*

The standard carbohydrate-rich meal was fed at 9 a.m. At 5 p.m. the rats were given 1 g. of the amino acid or 2 ml. of olive oil and were killed 2 hr. later. Control groups received no food at 5 p.m. Each entry is the mean glycogen concentration (±S.E.) in the liver. In one experiment with glycine, total carbohydrate concentration was measured in place of glycogen. The data from the fed and control groups were compared statistically by the *t* test. Probabilities less than 0·05 were taken to indicate a significant effect of the substance fed.

Substance fed	No. of rats per group	Liver glycogen (g./100 g. of liver)		Probability level for difference between groups
		Fed group	Control group	
Glycine	4	0·06±0·01	2·06±0·38	< 0·01
Glycine	5	*0·37±0·09	*2·01±0·37	< 0·01
DL-Alanine	4	0·72±0·22	2·29±0·38	< 0·02
L-Glutamic acid	4	1·04±0·21	2·25±0·36	< 0·05
Olive oil	3	2·34±0·18	2·36±0·42	> 0·05

* Total carbohydrate measured in place of glycogen.

Table 3. *Effect of casein or glycine administration on glycogen concentration in the livers of adrenodemedullated rats*

The rats were fed the standard carbohydrate-rich meal at 9 a.m. and the casein or glycine at 5 p.m. and were killed 2 hr. later. Control animals received no food at 5 p.m. Each entry is the mean (±S.E.) of observations on three rats (Expt. 1) or five rats (Expt. 2).

Expt. no.	Substance administered	Liver-glycogen concn. (g./100 g. of liver)
1	None	1·85±0·16
	Casein (2·5 g.)	1·15±0·17*
2	None	1·32±0·35
	Glycine (1 g.)	0·07±0·01†

* Significantly different from the control value ($P < 0·05$ by the *t* test).

† Significantly different from the control value ($P < 0·01$ by the *t* test).

series. When smaller amounts of casein were administered, the effect was correspondingly less. The occurrence of a dose–response relationship of this magnitude is rather surprising, since most of the casein was still in the stomach when the animals were killed 2 hr. after feeding.

Effect of feeding amino acids and fat. The action of casein could be reproduced by feeding individual amino acids, but not by feeding fat (Table 2). Only three amino acids, glycine, alanine and glutamic acid, proved palatable enough to be consumed quickly when they were fed individually to the rats in amounts of 1 g. Each caused a significant decrease in glycogen concentration, glycine being the most effective. The feeding of olive oil produced no alteration in liver-glycogen concentration.

In one of the experiments recorded in Table 2, the total carbohydrate concentration in the liver was measured in place of glycogen. After feeding glycine, there was a large decrease in total carbohydrate concentration, comparable in magnitude with the change observed in liver-glycogen content. This is consonant with the observation of Seifter *et al.* (1950) that total carbohydrate concentration in the liver is essentially the same as glycogen concentration under a variety of circumstances.

Action of casein and glycine on the liver glycogen of adrenodemedullated rats. Several investigators have shown that injection of glycine and other amino acids into rabbits produces a hyperglycaemia that can be abolished by adrenalectomy or by ergotamine administration (Pollak, 1922; Nord, 1926; Lundsgaard, 1930; Basiliou & Zell, 1931). These authors concluded that the injected amino acids caused release of adrenaline and thus mobilized liver glycogen. A similar mechanism could be responsible for the loss of liver glycogen observed by us after feeding protein or individual amino acids. An experiment was therefore carried out in which protein or an amino acid was fed to adrenodemedullated rats. Table 3 shows that both casein and glycine caused significant decreases in liver-glycogen concentration in these animals. The picture in the adrenodemedullated rat is thus similar to that observed in animals with intact adrenal glands.

Effect of casein and glycine administration on muscle-glycogen and blood-sugar concentration. In order to obtain more information about changes in carbohydrate metabolism after administration of protein or amino acids, blood-sugar and muscle-glycogen concentrations were measured at various times after feeding casein (Fig. 1) or glycine (Table 4). Within an hour of feeding casein, the concentration of glycogen in the liver had already fallen considerably (Fig. 1). This coincided with a significant decrease in blood-sugar concentration, which persisted throughout the 2 hr. period after feeding. On the other hand, glycogen concentration in muscle did not undergo any measurable alterations. A similar picture was obtained in animals fed with glycine 2 hr. before being killed

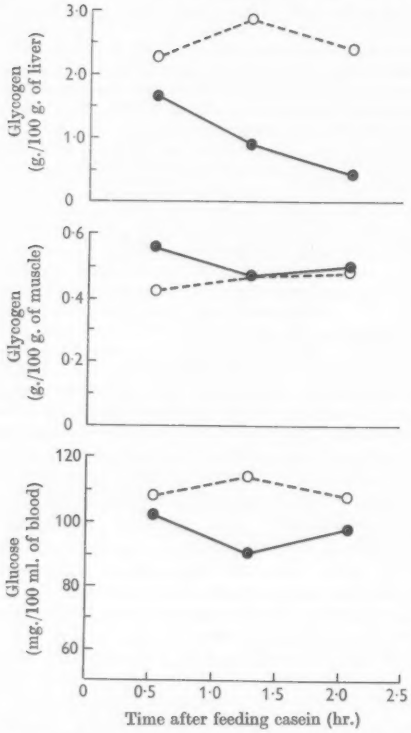

Fig. 1. Rats were given a meal of carbohydrate at 9 a.m. At 5 p.m. some of the animals (●) were fed with 2·5 g. of casein; control animals (○) received no food at this time. Rats from each group were killed thereafter at the times indicated. Each point is the mean of observations on five animals. Statistical analysis shows that liver-glycogen and blood-sugar concentrations were significantly lowered by casein administration ($P < 0.01$), but there were no significant alterations in muscle-glycogen concentration ($P > 0.05$).

(Table 4). The decrease in blood-sugar concentration after glycine administration was confirmed in a separate experiment in which the more specific glucose-oxidase procedure was used in place of the Hagedorn & Jensen (1923) method (Table 4).

The blood-sugar concentrations of the unfed control rats used in these experiments were appreciably higher than the concentrations normally observed in starving rats. This is presumably due to the carbohydrate meal that was fed 8–10 hr. before killing in order to cause glycogen deposition in the liver. Thus the control animals shown in Fig. 1 had a mean blood-sugar concentration of 109 ± 3 mg./100 ml., whereas a group of rats killed at similar times of day but not given the carbohydrate meal in the morning had a mean blood-sugar concentration of 88 ± 4 mg./100 ml. These animals had only traces of glycogen in their livers.

Effect of glycine administration on the liver glycogen of alloxan-diabetic rats. Since a decrease in blood-sugar concentration was observed after feeding protein or amino acids, it was possible that secretion of insulin might occur after giving these nutrients. There is some published evidence to support this possibility. In certain human subjects, administration of leucine causes a fall in blood-sugar concentration through release of insulin (Butterfield, Whichelow, Wright & Woolf, 1960). Insulin so released might cause the disappearance of glycogen from the liver; Levin & Weinhouse (1958) have observed that administration of insulin to rats in the post-absorptive state results in a loss of glycogen from the liver.

In order to test the possibility that insulin may participate in the action of protein and amino acids on carbohydrate metabolism, rats were made diabetic with alloxan and were then fed with glycine. A control group of diabetic rats were fed the same carbohydrate-rich meal in the morning, but received no glycine at 5 p.m.; the glycogen concentrations in the livers of these control

Table 4. *Glycogen content of liver and muscle and the blood-sugar concentration after feeding glycine*

The rats were given a standard carbohydrate-rich meal at 9 a.m. At 5 p.m. they were fed 1 g. of glycine and were killed 2 hr. later. Control rats received no food at 5 p.m. Each entry is the mean (±S.E.) of observations on four rats except for the determinations of blood-sugar concentration by the glucose-oxidase procedure, which were carried out on a separate series of animals with eight rats in the control and glycine-fed groups.

Substance administered	Liver glycogen (g./100 g. of liver)	Muscle glycogen (g./100 g. of muscle)	Blood sugar (mg./100 ml. of blood) Hagedorn–Jensen method	Blood sugar (mg./100 ml. of blood) Glucose-oxidase method
None	2·13±0·39	0·45±0·04	107±3	123±5
Glycine	0·61±0·42†	0·48±0·12*	83±6‡	99±6‡

* Not significantly different from the control value ($P > 0.05$ by the t test).
† Significantly different from the control value ($P < 0.05$ by the t test).
‡ Significantly different from the control value ($P < 0.01$ by the t test).

Table 5. *Effect of glycine administration on glycogen concentration in the livers of alloxan-diabetic rats*

The rats were fed the standard carbohydrate-rich meal at 9 a.m. and 1 g. of glycine at 5 p.m. and were killed 2 hr. later. Control animals received no food at 5 p.m. Each entry is the mean (±S.E.) of observations on five rats. Glycine administration caused a significant decrease in liver-glycogen concentration ($P < 0.05$ by the t test).

Substance administered	Liver-glycogen concn. (g./100 g. of liver)	Blood-sugar concn. (mg./100 ml. of blood)
None	1·87±0·51	285±21
Glycine	0·31±0·17	250±23

animals (Table 5) were similar to those observed in non-diabetic control rats fed in the same way (Tables 1–4). This confirms previous observations that fed diabetic rats have a normal concentration of glycogen in their livers (Renold, Teng, Nesbett & Hastings, 1953). When glycine was administered to the diabetic rats, there was a decrease in liver-glycogen concentration similar in magnitude to the effect observed in non-diabetic animals, indicating that an intact insulin-secreting mechanism is not essential for the action of glycine. Blood-sugar concentrations are also quoted in Table 5, to show that the animals studied were diabetic; wide variations between individual animals make it impossible to decide whether glycine administration lowered the blood-sugar concentration significantly.

DISCUSSION

When rats with high concentrations of liver glycogen are fed with protein or with single amino acids, there is a rapid and considerable fall in the amount of glycogen and of total carbohydrate in their livers (Tables 1 and 2). Previous experiments (Clark *et al.* 1960) show that a decrease in adenosine triphosphate in the liver accompanies this loss of glycogen.

Our data appear to exclude the possibility that administration of protein or amino acids causes glycogenolysis as the result of adrenaline release, since loss of liver glycogen occurs after feeding casein or glycine to adrenodemedullated animals (Table 3). Release of adrenaline is also incompatible with our finding (Fig. 1 and Table 4) that the disappearance of glycogen from the liver after protein or glycine administration is not accompanied by a rise in blood-sugar concentration or by a fall in muscle glycogen, both characteristic features of the action of adrenaline (Sokal & Sarcione, 1959). Release of glucagon, though not excluded as part of the mechanism, would appear to be an unlikely reason for the loss of glycogen from the liver in view of the absence of a rise in blood-sugar concentration (Foà, Galansino & Pozza, 1957).

Insulin can induce glycogenolysis in the liver under some circumstances. Levin & Weinhouse (1958) produced glycogen deposition in the livers of rats by feeding them with glucose. When insulin was then injected, the fall in blood-sugar concentration was accompanied by a rapid loss of glycogen from the liver, but there was no alteration in glycogen concentration in muscle. The picture we have obtained after feeding casein (Fig. 1) or glycine (Table 4) is identical with this. Nevertheless, secretion of insulin does not appear to be part of the mechanism by which protein and amino acids cause loss of liver glycogen, since this effect still occurs in alloxan-diabetic animals (Table 5).

Whatever may initiate the mechanism of glycogen breakdown in the liver, the utilization of the products of breakdown is of interest in view of the specific dynamic action of absorbed amino acids. The amino acids found by us to cause loss of liver glycogen are all effective in inducing a specific dynamic action irrespective of the route of administration (Weiss & Rapport, 1924; Nord & Deuel, 1928; Lundsgaard, 1931; Wilhelmj, Bollman & Mann, 1931). This specific dynamic action occurs very soon after feeding the amino acids and would thus coincide with the loss of liver glycogen observed in our experiments. A causal relationship is suggested by experiments in which Wilhelmj & Mann (1930) injected glycine or alanine into dogs that had been previously fed on different types of diet. When the animals had been without food for some days, injection of the amino acids caused a specific dynamic action that was unaccompanied by any change in R.Q. However, when the dogs had been on a standard mixed diet and notably when they had been receiving a diet rich in carbohydrate, administration of the amino acids caused a rise in R.Q., indicating preferential combustion of stored carbohydrate during the period of specific dynamic action.

The amount of glycogen lost from the liver of the rat after glycine administration can be compared quantitatively with the additional energy released by the specific dynamic action of this amino acid. The data in Table 4 show that, 2 hr. after feeding 1 g. of glycine, liver-glycogen concentration was lowered by 1·5 g./100 g. of liver below that observed in the control group. Since female rats of the size used (170 g.) have livers weighing about 5 g. (Goodlad & Munro, 1959), the total quantity of glycogen lost from the liver thus amounts to some 75 mg., and on combustion this would yield 0·3 kcal. The specific dynamic action of glycine has not been measured on rats under conditions identical with those of our experiments, but Lewis & Luck (1933) fed approximately 1 g. of glycine to fasting

rats weighing 200 g. and observed an elevation of energy output lasting about 2·5 hr. and amounting to 0·32 kcal. Thus the amount of energy dissipated by the specific dynamic action of glycine could account for all the glycogen lost from the liver. This explanation is compatible with the decrease in adenosine triphosphate concentration which occurs at the same time as the loss of liver glycogen after casein administration (Clark *et al.* 1960). The coincident decrease in blood-sugar concentration (Fig. 1 and Table 4) is presumably caused by the depletion of liver glycogen, since our data show that loss of liver glycogen as the result of a prolonged lack of food is also accompanied by a fall in blood-sugar concentration.

SUMMARY

1. Rats were given a meal of carbohydrate which caused deposition of glycogen in their livers, and were then fed on casein, glycine, alanine, glutamic acid or olive oil.

2. The casein and the amino acids each caused a large loss of glycogen from the liver. This action was already considerable within 1 hr. of administration. There was no change in glycogen concentration 2 hr. after feeding olive oil.

3. Coincident with the loss of liver glycogen after protein or amino acid administration, there was a fall in blood-sugar concentration. There was no change in muscle-glycogen concentration.

4. The feeding of protein or amino acids to adrenodemedullated rats or to alloxan-diabetic rats resulted in a loss of glycogen from their livers. It was concluded that secretion of adrenaline or of insulin is not an essential part of the mechanism responsible for loss of liver glycogen after protein administration.

5. It is suggested that the loss of liver glycogen after administration of protein or amino acids is associated with the extra energy expended during the specific dynamic action of the absorbed amino acids.

It is a pleasure to acknowledge the kindness of Dr R. S. Crossland in supplying us with adrenodemedullated rats. Histological examination of the glands to confirm the completeness of the removal of the medulla was carried out by Mr F. Weston. Miss M. Mathieson gave skilled assistance with many of the glycogen and blood-sugar determinations. The Medical Research Council generously provided a grant to one of us (G.A.J.G.) for scientific assistance and for materials used in the course of the investigations.

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A Note on the Estimation of Dehydro-L-Ascorbic Acid in Plant Tissues by the Roe & Kuether Procedure

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Dehydro-L-ascorbic acid is usually found in plant tissues in low concentrations and is always associated with ascorbic acid in much higher concentrations. One of the most reliable and direct methods for its estimation is that originally proposed by Roe & Kuether (1943) which is based on the formation of a 2:4-dinitrophenylosazone.

Many plant tissues, however, contain so little dehydro-L-ascorbic acid that after extraction and dilution with the phenylhydrazine reagents the extracts may contain less than 0·5 μg. of dehydro-L-ascorbic acid/ml. and even lower values may be encountered if the tissue has been exposed to anaerobic conditions. If more concentrated extracts are prepared interference is often observed on the addition of the 85 % sulphuric acid reagent, owing to formation of coloured products. A more serious disadvantage, and one which makes it impossible to use the method at all, is that with some plant extracts hydrazones of other compounds are formed which absorb strongly in the same region (530 mμ) as the red 2:4-dinitrophenylosazone formed from dehydro-L-ascorbic acid. The method has been modified to permit of the more accurate estimation of dehydro-L-ascorbic acid when present in small amounts and to effect the separation of the osazone of dehydro-L-ascorbic acid from other hydrazones formed from other compounds in plant tissues.

REAGENTS

2:4-Dinitrophenylhydrazine, metaphosphoric acid and thiourea were analytical-grade reagents. The acid-activated alumina (Woelm) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. For routine estimation of dehydro-L-ascorbic acid (DHA) in plant tissues the dinitrophenylosazone formed from DHA was separated from other hydrazones or osazones on columns (5 cm. × 1 cm.) of alumina.

RESULTS

Concentration of the osazone. The red dinitrophenylosazone formed from DHA is almost insoluble in the sulphuric acid–phenylhydrazine reagent and may be quantitatively collected from extracts by centrifuging or filtration or both. By this means the total DHA from 100 g. or even larger amounts of tissue may be conveniently prepared. In this way the osazone may be concentrated, and at the same time separated from unchanged phenylhydrazine and many other undesirable constituents of the tissue.

After extraction of the plant tissue, carried out by the methods described by Barker & Mapson (1959) to avoid any oxidation of ascorbic acid during extraction, the usual procedure of Roe & Kuether is followed whereby the extract is incubated with 2:4-dinitrophenylhydrazine in the presence of thiourea for 3 hr. at 37°. When microgram quantities of DHA are present with milligram quantities of ascorbic acid it is advisable and even essential to remove all O_2 from the solution before incubation with the phenylhydrazine reagent.

Procedure. The tissue is extracted with metaphosphoric acid (5 %, w/v) at −3° under anaerobic conditions (Barker & Mapson), and to this extract 4 ml. of 2:4-dinitrophenylhydrazine (2 %, w/v) in 9N-H_2SO_4 is added together with 200 mg. of thiourea to every 20 ml. of extract. The solution is freed from O_2 by bubbling with N_2 and is then incubated for 3 hr. at 37°.

After incubation the solution is centrifuged at 20 000*g* for 30 min. to aggregate the insoluble hydrazones and osazones. The supernatant is passed through sintered-glass filters, and any precipitate collected is combined with that centrifuged down. The combined precipitates are washed with 0·1N-H_2SO_4 to remove most of the unchanged 2:4-dinitrophenylhydrazine reagent. The washed hydrazones are dissolved in a small volume of ethyl acetate, and the solution is transferred to a separating funnel. If, as is the case with many plant extracts, hydrazones of the α-oxo acids are also present, these may with advantage be removed at this stage by extracting the ethyl acetate solution twice with 0·2M-$NaHCO_3$. By this treatment these latter hydrazones are rendered soluble in the aqueous phase, whilst the red osazone formed from DHA remains in the non-aqueous phase. The ethyl acetate solution is washed again with 0·1N-H_2SO_4, and, after carefully separating the aqueous phase, the ethyl acetate solution is concentrated *in vacuo* to dryness; the hydrazones are dissolved in 1 ml. of ethyl acetate to which is added 5 ml. of H_2SO_4 (50 %, w/v). The concentration of the red osazone is determined from the difference between the absorption at 530 mμ of this solution and a similar solution in which the DHA had been previously reduced by H_2S in the usual Roe & Kuether method. Dehydroascorbic acid standards (prepared by the oxidation of ascorbic acid with Br_2) are prepared and are carried through the same procedure.

Tests have been carried out with the above procedure with the osazone prepared from DHA present in amounts varying from 5 to 75 μg. in 20 ml. of solution. Fig. 1 shows that, after the standard procedure described above, a linear relation exists between the amounts of DHA taken and the amount of osazone collected over the range 10–75 μg. of DHA/20 ml. of solution. With less than 10 μg. of DHA there was a departure from this linear relationship. This we attribute mainly to a small loss of the osazone due to a low but finite solubility in the reagent solution; this loss appears to be constant under our standard conditions. Fortunately with this procedure more concentrated extracts may be prepared so as to increase the concentration of the osazone, and we suggest that a suitable amount of tissue should be extracted to yield, if possible, an extract containing not less than 0·5 μg. of DHA/ml.

Separation on acid alumina. With some plant tissues (e.g. peas) the absence of compounds forming hydrazones or osazones or both which interfere in the estimation makes it possible to proceed simply as described above. With other plant tissues (strawberry leaves) this procedure is inapplicable since during the treatment with 2:4-dinitrophenylhydrazine other hydrazones or osazones or both are formed which absorb strongly in the region of 530 mμ and cause serious interference.

It is possible to separate these compounds from that of the red osazone formed from DHA by chromatography on acid-activated alumina. Columns of acid alumina are prepared with ethyl acetate containing 2 % (v/v) of acetic acid, the hydrazones from the tissue are added in solution in ethyl acetate, and the chromatogram is developed with the ethyl acetate–acetic acid solvent. Any unchanged 2:4-dinitrophenylhydrazine is first eluted but this need not be separated, unless desired, from the following band of the red osazone of DHA which is readily eluted from the column by the ethyl acetate–acetic acid reagent. Other hydrazones are firmly held on the column, and include those formed from α-oxo acids, which makes it unnecessary to use the extraction with $NaHCO_3$ described above if separation on alumina is employed.

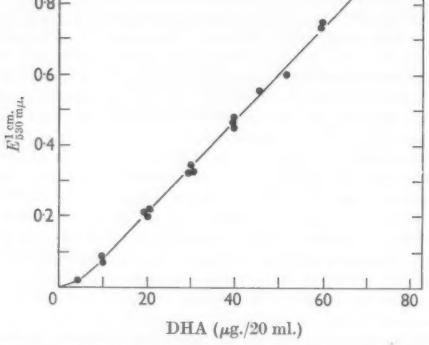


Fig. 1. Relation between the concentration of DHA and the dinitrophenylosazone formed.

The eluates are concentrated *in vacuo* and the concentration of the osazone formed from DHA is determined as described above. Tests carried out with solutions of the osazone prepared from DHA show that the osazone is quantitatively recovered from the column, if this is well washed with the ethyl acetate–acetic acid reagent. With this method good recoveries of DHA added to extracts of potatoes and strawberry leaves have been obtained (Table 1). It is also possible to separate quantitatively the osazone formed from DHA by paper chromatography, by using an aqueous solvent containing phenol (10 %, w/v), acetic acid (10 %, v/v) and glycerol (10 %, v/v), and by eluting the osazone from the appropriate area of the chromatogram with ethyl acetate. This method possesses no advantage over that outlined above and is more tedious to operate. Analysis by paper chromatography can, however, serve as a valuable qualitative check on the results obtained by the alumina method.

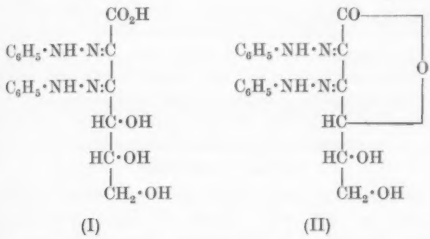
Formation of open- and closed-chain osazones from dehydroascorbic acid

Herbert, Hirst, Percival, Reynolds & Smith (1933) first pointed out the numerous possibilities for structural isomerisim and stereoisomerism of the phenylhydrazine derivatives of DHA. Hirst (1933) noted the presence of two different phenylosazones obtained from the oxidized form of DHA

Table 1. *Accuracy of the method as determined by the estimation of known amounts of dehydro-*L*-ascorbic acid added to plant extracts*

Plant extract	DHA added (μg./20 ml. of extract)	DHA estimated (μg./20 ml. of extract)	Error (%)
Strawberry leaf	30	32	+6
	50	52	+4
	20	19	−5
	20	21	+5
	30	30	0
	20	18	−10
	30	29	−3
Potato	20	19	−5
	20	21	+5
	40	39	−2·5

and phenylhydrazine and Ohle & Bockman (1934) found that, in the presence of mineral acids, osazones corresponding to the open- and closed-chain forms of dehydroascorbic acid (I and II) could be obtained.



(I) (II)

In our experiments we have also found evidence that two similar osazones are formed as a result of the reaction between 2:4-dinitrophenylhydrazine and DHA under the conditions of the Roe & Kuether procedure. These two forms have been separated by paper chromatography by using as solvent the phenol–acetic acid–glycerol mixture. The open-chain compound, an orange–yellow form with R_F 0·43, is easily separated from the red lactone form with R_F 0·66. These two compounds may also be separated on the alumina column in the manner described above, under which conditions the orange–yellow component is adsorbed strongly on the alumina and may, after removal of the red component with the ethyl acetate–acetic acid solvent, be eluted with acetone containing 0·2M-oxalic acid. After elution and on standing (24 hr. at 20°) in the presence of oxalic acid the orange–yellow compound is completely converted into the red component as shown by the change, revealed by paper chromatography, from an orange–red component with R_F 0·43 to a red component with R_F 0·66. A similar occurrence was reported by Probst & Schultze (1950) after chromatography of 2:4-dinitrophenylhydrazine derivatives on magnesium phosphate columns.

The red lactone form isolated after chromatography on alumina and recrystallized from an ethanol–acetone mixture (1:1) appears to be identical with the derivative prepared by the above workers, giving in 9N-H_2SO_4 an absorption spectrum with maxima at 525 and 370 mμ and in which the ratio of the extinctions measured at 530:450, 370:450, and 370:530 were 2·1, 2·25 and 1·08 respectively, values which are almost identical with those reported by Probst & Schultze.

The lactone form may be reconverted into the open-chain form by shaking an ethyl acetate solution of the red derivative with 0·1N-NaOH, when the orange–yellow compound formed becomes soluble in the aqueous phase and may be recovered, after acidification with acetic acid, by extraction into ethyl acetate. These changes have likewise been demonstrated by paper chromatography.

Fortunately, from the point of view of estimating DHA, the formation of the red osazone predominates, under our conditions, over that of the orange–yellow osazone. The relative amounts of the two compounds formed have been estimated after separation on alumina and conversion of the open-chain form into the lactone form. Under the standard conditions used here for the formation of the osazone, 97–98 % of the total osazone formation is in the lactone form. Since, moreover, the amount of the lactone form is proportional to the original concentration of DHA present, the failure to estimate the small amount of the orange–yellow component is of little consequence as far as the estimation of DHA is concerned.

SUMMARY

1. A modification of the Roe & Kuether (1943) procedure for the estimation of dehydroascorbic acid in plant tissues is described. The dinitrophenylosazone formed from dehydroascorbic acid may be separated from other interfering hydrazones or osazones by chromatography on acid alumina.

2. Evidence of the formation of an open-chain osazone and of a lactone form has been obtained when dehydroascorbic acid reacts with 2:4-dinitrophenylhydrazine under the conditions of the Roe & Kuether test.

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An Enzymic Method for the Estimation of L-Tryptophan

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Strictly specific, but time-consuming, microbiological assay procedures for L-tryptophan have been published (Dunn, Schott, Frankl & Rockland, 1945; Schweigert, Sauberlich & Elvehjem, 1946). The chemical method of Udenfriend & Peterson (1957), with nitrite and *p*-dimethylaminobenzaldehyde, lacks specificity. Egelhaaf (1957) has reported a very sensitive chromatographic method, in which the tryptochrome complex is measured fluorimetrically. The procedure is rather lengthy and has an error of $\pm 10\%$.

The enzymic determination described here is strictly specific for L-tryptophan, suitable for quantities as low as 1 μg. and fairly rapid.

L-Tryptophan is converted almost quantitatively into indole, pyruvic acid and ammonia by the enzyme tryptophanase under optimum conditions. The indole is determined colorimetrically with *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamic aldehyde, after extraction from incubation mixtures with light petroleum (b.p. 40–60°). This indole determination is a modification of the original method of Happold & Hoyle (1934) for the measurement of indole in bacterial cultures.

MATERIALS AND APPARATUS

Extraction funnels. These funnels (250 ml. capacity) should be of a narrowly tapering pear shape, so that a 40 ml. graduation mark can be made accurately.

Tryptophanase preparation. An acetone-dried powder of *Escherichia coli* cells possessing tryptophanase activity was prepared according to Dawes, Dawson & Happold (1947). The acetone-dried cells keep indefinitely at $-15°$. The acetone-dried powder (0·2 g.) was extracted at room temperature for 1 hr. with 10 ml. of water and centrifuged, and the clear, straw-coloured supernatant retained.

Pyridoxal phosphate. This was an aqueous 0·02% solution, stored in the dark at 4°.

Buffer. KH_2PO_4 (18 g./l.) was adjusted to pH 7·8 with 5 N-NaOH.

Trichloroacetic acid. This was an aqueous 50% (w/v) solution.

Dimedone. This was an ethanolic 1% (w/v) solution.

p-*Dimethylaminobenzaldehyde reagent.* A.R. *p*-dimethylaminobenzaldehyde (16 g.) was dissolved in 1·25 l. of absolute ethanol and 320 ml. of conc. HCl.

p-*Dimethylaminocinnamic aldehyde reagent.* *p*-Dimethylaminocinnamic aldehyde (2·34 g.) was dissolved in 780 ml. of ethanol and 100 ml. of conc. HCl and water to 1 l. (Turner, 1959).

EXPERIMENTAL AND RESULTS

Partition of indole between water and light petroleum

Standard, aqueous indole solutions (25 ml.) were shaken at 20° with 25 ml. of light petroleum (b.p. 40–60°). Equilibration was complete in 30 sec. Indole was determined in the aqueous phase by the absorption at 275 mμ (Unicam SP. 500 spectrophotometer, 1 cm. light-path), with water equilibrated with an equal volume of light petroleum at the blank. The calibration curve was linear up to an extinction of 0·84, which corresponded to 1·84 mg. of indole/100 ml.

The results shown in Table 1 give an average value of 5·01 for the partition coefficient in favour of the light petroleum. About 17% of the indole is left in the aqueous phase and so standardization of the volumes of aqueous and organic phases is necessary during the extraction procedure.

Reaction of indole with pyridoxal phosphate

All incubation mixtures contain pyridoxal phosphate as the coenzyme of tryptophanase. Under the acid conditions produced when trichloroacetic acid is used to precipitate the protein, indole and pyridoxal phosphate (or pyridoxal) react to form a red pigment. This loss of indole can be prevented by the addition of dimedone, which combines with the pyridoxal phosphate and renders it unavailable for reaction with the indole.

Table 1. *Partition coefficient of indole between light petroleum and water*

Aqueous indole was equilibrated at 20° with an equal volume of light petroleum (b.p. 40–60°); indole in the aqueous phase was determined by absorption at 275 mμ.

Initial concn. of indole in aq. phase (mg./100 ml.)	Concn. of indole in aq. phase after equilibration (mg./100 ml.)	Concn. of indole in light petroleum after equilibration (calc. by difference) (mg./100 ml.)	Partition coeff.
0·53	0·09	0·44	4·88
0·98	0·16	0·82	5·13
1·65	0·27	1·38	5·11
1·65	0·28	1·37	4·93
		Average partition coefficient	5·01

The exact nature of the red pigment formed from indole and pyridoxal phosphate is unknown, but phosphate is absent.

Colorimetric reagents

Formation of the 'roseindole' colour between indole and *p*-dimethylaminobenzaldehyde (Fischer, 1886) is complete within a few seconds. After reaching a peak value, the extinction (measured at $\lambda_{max.}$ 570 mμ, see Fig. 1) decreases at the rate of 2·9 %/min. (see Fig. 2). In practice, the colour formed was read exactly 5 min. after adding this reagent.

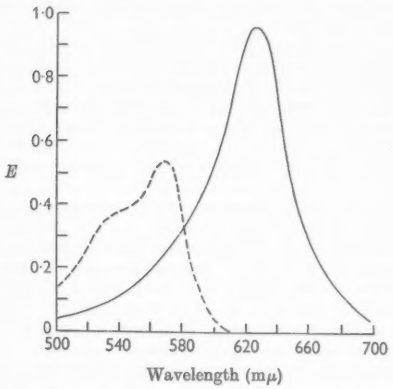

Fig. 1. Absorption spectra of 'roseindoles'. – – –, Indole (32 μg.) extracted from aqueous solution with equal volume of light petroleum (b.p. 40–60°) and colour developed with 10 ml. of *p*-dimethylaminobenzaldehyde reagent, $\lambda_{max.}$ 570 mμ, scanning time 5 min.; ——, indole (22 μg.) treated similarly and colour developed with *p*-dimethylaminocinnamic aldehyde reagent, $\lambda_{max.}$ 625 mμ, read after 15 min.

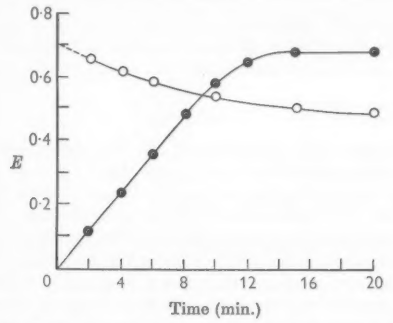

Fig. 2. Development and decay of indole colour complexes at 20°. ●, Development of green colour produced from indole and *p*-dimethylaminocinnamic aldehyde reagent, measured at 625 mμ; ○, decay of red colour produced from indole and *p*-dimethylaminobenzaldehyde reagent, measured at 570 mμ.

p-Dimethylaminocinnamic aldehyde produces a green colour ($\lambda_{max.}$ 625 mμ). Colour development is complete in 15 min. and the maximum intensity remains constant for several hours (see Fig. 2).

With the same amount of indole, the intensity of absorption (at 625 mμ) with the latter reagent is approximately 2·5 times that with the former (at 570 mμ).

Procedure

Equal volumes of pyridoxal phosphate solution and tryptophanase preparation are incubated for 10 min. at 37°. This mixture (2 ml.) is added to 3 ml. of test tryptophan solution and 5 ml. of phosphate buffer.

Incubation is continued for 30 min. at 37°. Dimedone reagent (1 ml.), followed by 5 ml. of trichloroacetic acid solution, is then added. After 3–4 min., the coagulated protein is filtered off through a plug of absorbent cotton wool in a 1 in. diameter glass funnel. The filtrate passes directly into 40 ml. of light petroleum contained in an extraction funnel, and the incubation tube and filter are washed with water until the aqueous layer in the funnel reaches the 40 ml. graduation mark. Organic and aqueous phase are shaken vigorously together for 30 sec. and allowed to separate. The bottom, aqueous layer is run off and, to avoid error, any residual water on the sides of the funnel is removed by repeating the shaking process.

p-Dimethylaminobenzaldehyde reagent (10 ml.) or *p*-dimethylaminocinnamic aldehyde reagent (10 ml.) is added to the light-petroleum layer and shaken vigorously for a few seconds. The lower, coloured reagent layer is removed and, if necessary, clarified by low-speed centrifuging or rapid filtration through absorbent cotton wool.

Colours are read at $\lambda_{max.}$ against a reagent blank (Unicam SP. 600 spectrophotometer, 1 cm. light-path).

With *p*-dimethylaminobenzaldehyde reagent, the standard curve is linear up to *E* 0·6 at 570 mμ, which corresponds to 60 μg. of L-tryptophan. With *p*-dimethylaminocinnamic aldehyde reagent, the standard curve is linear up to *E* 0·76 at 625 mμ, which corresponds to 30 μg. of L-tryptophan.

Error

Twenty separate estimations were performed, each on a different day, with the same standard L-tryptophan solution of 60 μg. of L-tryptophan/3 ml., with the *p*-dimethylaminobenzaldehyde reagent. The mean value for L-tryptophan was 59·8 μg., with an S.D. of $\pm$ 2·22 μg.

DISCUSSION

The method was originally developed for the measurement of L-tryptophan in growing bacterial cultures. It has since been used successfully for determining L-tryptophan in the intracellular, free amino acid pool of *Escherichia coli* and in *Calliphora erythrocephala* during development from larva to imago (Scott, 1960), and in protein hydrolysates (Toothill, 1961), so it appears generally applicable to biological material. Tryptophanase is a sulphydryl enzyme (Happold & Turner, 1960) and, when oxidizing agents are present in the solution under test it would seem expedient to protect the enzyme by the addition of glutathione. This has never been found necessary, however, by the author.

Tryptophanase is strictly specific for L-tryptophan and converts no other known, naturally occurring compound into indole or indole derivatives. If test solutions already contain indole (or other substances soluble in light petroleum and capable of colour production with either of the colorimetric reagents), this can be removed first by light-petroleum extraction.

SUMMARY

1. An enzymic method is described for the estimation of L-tryptophan in biological material, in the range 1–60 μg.

2. L-Tryptophan is converted quantitatively into indole by the enzyme tryptophanase, and the indole measured colorimetrically, after extraction with light petroleum, with either *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamic aldehyde reagents.

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Phospholipids in Ram Semen: Metabolism of Plasmalogen and Fatty Acids

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Previous studies on the nature and function of lipids in ram semen led us to conclude that the phospholipids of spermatozoa consist mainly of choline-based plasmalogen, and that sperm respiration in the absence of seminal fructose is largely dependent on the oxidation of fatty acids that arise from hydrolysis of this plasmalogen (Lovern, Olley, Hartree & Mann, 1957; Hartree & Mann, 1959, 1960*a*).

The present paper is an extension of these studies, the results of which fall under three headings: (1) A comparison has been made between the components of the phospholipid fraction extracted from freeze-dried spermatozoa and those of corresponding fractions obtained from fresh, or frozen and thawed, spermatozoa. This comparison was prompted by the appearance of papers by Gray (1960*a*) and by Wren (1960). Gray produced evidence that the phosphatide fraction extracted from frozen whole ram semen contained lecithin and plasmalogen in approximately equal amounts. Wren drew attention to the widespread occurrence in tissues of lipoamino acids and lipopeptides and concluded that such lipid derivatives may be artifacts when prepared from freeze-dried material. (2) Direct quantitative evidence for the

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role of fatty acids in the metabolism of washed spermatozoa has been obtained by analysis of the free fatty acids extractable from spermatozoa. Changes in fatty acid content have been correlated with simultaneous decreases in the plasmalogen of the sperm phospholipids. (3) The action of different components of ram semen upon the respiratory activity of spermatozoa has been studied with particular reference to previous reports of stimulatory effects by lecithin (Lardy & Phillips, 1941*a*, *b*; Lardy, Hansen & Phillips, 1945; Bomstein & Steberl, 1957; Iida, 1958).

MATERIALS

Ram semen (pooled ejaculates of ten rams) was collected by means of an artificial vagina (Walton, 1945) and spermatozoa were separated from seminal plasma by centrifuging. Washed sperm suspensions were prepared with a calcium-free 'Ringer solution for spermatozoa' (Mann, 1946). Two procedures have been followed for separation of spermatozoa and plasma: both were carried out at room temperature.

Wash procedure 1. To prevent damage to the cells, spermatozoa required for metabolic experiments were washed by centrifuging at low speed. Semen was diluted to 5 vol. with Ringer solution and centrifuged for 15 min. at 1300*g* (mid-point of tube). The upper fluid was removed and the cell mass resuspended to 5 vol. in Ringer solution. The suspension was centrifuged as before and the cells were resuspended to 2–3 times the original semen volume in Ringer solution. The loss of spermatozoa in the wash fluids averaged 6–7%.

Wash procedure 2. For analytical studies of intracellular lipids, spermatozoa were separated more completely from plasma by using higher centrifuge speeds. Undiluted semen (1 vol.) was centrifuged for 15 min. at 20 000*g*. The plasma was removed as completely as possible, and the cells were resuspended to 5 vol. and again centrifuged at 20 000*g*. The washed cells were resuspended to the original semen volume.

Seminal plasma. The sperm-free seminal plasma, obtained by wash procedure 2, was used either as such or after dialysis in cellophan tubing. Seminal plasma (10 ml.) was dialysed at 5° against two lots of 0·9% NaCl and finally against Ringer solution (500 ml. in each case).

METHODS

Extraction and fractionation of lipids from freeze-dried, fresh, and frozen and thawed spermatozoa

The composition of all solvent mixtures is given on a v/v basis.

Extraction of phospholipids from freeze-dried spermatozoa. During the period December–April several batches of ram sperm were freeze-dried immediately after they had been washed by procedure 2, and stored at −15° until the following January when the lipids were extracted. The freeze-dried, powdered material (20 g.) was treated in five portions as follows. Each portion (4 g.) was suspended in 16 ml. of water and homogenized in a Waring Blendor for 5 min. with 400 ml. of chloroform–methanol (2:1). The products were filtered, and the filtrates were combined, emulsified in the Blendor with 0·2 vol. of 3 mM-$MgCl_2$ (Folch, Lees & Sloane-Stanley, 1957) and stored overnight at 5°. The upper phase was discarded, the chloroform phase was evaporated, the residue extracted with dry chloroform and the extract re-evaporated. The gummy product was dissolved in a little ether and phospholipids were precipitated by adding 11 vol. of acetone and storing for 48 hr. at 5°. The solid was collected, reprecipitated twice more with acetone and dissolved in chloroform.

Extraction and separation of lipids from fresh or frozen and thawed spermatozoa. Spermatozoa were washed by procedure 2 and either used immediately or stored for different periods at −15°. Of such suspensions 30 ml. was run slowly into 340 ml. of methanol in a 1 l. volumetric flask. The mixture was diluted with 200 ml. of chloroform, heated to the boiling point, cooled, made up to the mark with chloroform, mixed and filtered. The filtrate was washed with $MgCl_2$ solution as described above. Separation of phospholipids from other lipids was effected on a silicic acid column. The column was made up in chloroform as described by Hanahan, Dittmer & Warashina (1957) and contained 2·0 g. of silicic acid (Mallinckrodt; 100-mesh; analytical reagent suitable for chromatographic analysis) and 1·0 g. of Hyflo SuperCel (Johns-Manville Co. Ltd.)/mg. of applied lipid phosphorus. The lipid was applied in chloroform and the column developed with the same solvent until the effluent no longer gave the Liebermann–Burchard reaction for cholesterol (Cole, 1933).

The fatty acids were isolated from the chloroform effluent by fractionation on a column of Amberlite IRA-400. The procedure of Carlson & Wadström (1958) was followed except that the length and diameter of the column were both increased about threefold in order to accommodate approximately ten times the quantity of fatty acid used by Carlson & Wadström.

The phospholipids retained on the silicic acid columns were eluted with a series of chloroform–methanol mixtures of increasing methanol concentration and finally with pure methanol. The effluent was fractionated with a drop-counting fraction collector. The rate of flow was maintained, by means of a slight positive pressure of N_2, at approximately $2a$ ml./min. where a is cross-sectional area (cm.²) of the column. The fraction volumes increased as the effluent became richer in methanol, the mean volumes being $2a$ ml. Progress was followed by analysis of the fractions for phosphorus (King, 1932). The choline phosphatides, i.e. lecithin plus choline plasmalogen, were eluted with chloroform–methanol (2:1 or 3:2). The various fractions were stored in chloroform at −15° pending analysis.

Fractionation of wash-fluid obtained during the preparation of washed sperm suspensions

Washings from procedure 2 were accumulated in the frozen state and batches of 500 ml. were melted and concentrated *in vacuo* to 10 ml. This concentrate was used as follows. One part (15 ml.) was extracted with 30 vol. of chloroform–methanol (2:1) to yield 'total lipid' and insoluble material which was suspended in a little Ringer solution and dialysed exhaustively in cellophan tubing against Ringer solution to give the 'non-lipid, non-diffusible fraction'. A further 30 ml. of concentrate was dialysed against Ringer solution to give the 'dialysed concentrate', and half of this material was treated with chloroform–methanol to give 'dialysed total lipid'.

Estimation of total and individual fatty acids in lipids extracted from fresh and incubated spermatozoa

Aerobic and anaerobic incubation of washed spermatozoa. Suspensions of ram spermatozoa in Ringer solution (50 ml.), obtained by wash procedure 1, were treated with penicillin (50 000 units) and divided into three equal portions. One portion (*A*), the control, was extracted with lipid solvents immediately, another (*B*) was incubated anaerobically at 37°, and the third portion (*C*) was incubated aerobically at 37° by being shaken in Barcroft differential manometers, in the presence of NaOH for absorption of CO_2, which allowed O_2 consumption to be measured. Incubation periods were between 2·5 and 3 hr. (except in Table 6). Details of the methods have been described by Hartree & Mann (1959).

Extraction of lipids. Each of the portions *A*, *B* and *C* (15 ml.) was run slowly into 500 ml. volumetric flasks containing 170 ml. of methanol. Chloroform (100 ml.) was added to each flask and the mixtures were heated to the boiling point. After cooling, the suspensions were made up to the mark with chloroform and filtered. Non-lipid impurities were removed by shaking the extracts with 0·2 vol. of 0·5 M-NaH_2PO_4 (Carlson & Wadström, 1958). The extracts were evaporated and the lipids dissolved in chloroform (lipids *A*, *B* and *C*).

Determination of total free fatty acids. Carlson & Wadström (1958) separated free fatty acids from the total lipid of blood plasma by two-stage column chromatography. Thus on passing a chloroform solution of lipid through a silicic acid column (as above), phospholipids were retained. The non-absorbed lipid, in ether–ethanol, was passed through a column of Amberlite IRA-400 (OH) when only free fatty acids were retained. The latter were then eluted with ether–ethanol–acetic acid, esterified and estimated by the iron–hydroxamate reaction. In the present work difficulty was encountered in finding a solvent-proof closure for the bottoms of the Amberlite columns. Teflon and glass needle valves (Manostat Corp., New York City) proved to be satisfactory. For analysis of lipids *A*, *B* and *C*, the following modifications were made in the procedure described by Carlson & Wadström (1958): (1) In view of the high phospholipid content of ram-sperm lipid the length of the silicic acid columns was doubled and the volume of chloroform used for eluting neutral lipids plus fatty acids was increased by 50%. (2) The fatty acids were esterified with diazomethane instead of methanolic $SOCl_2$: in our hands the latter method was unreliable. Esterification was carried out by treating the fatty acid (0–4 μequiv.) with 2 ml. of 2% (w/v) diazomethane in ether and 0·2 ml. of methanol at 20°. After 5 min. the solvents were evaporated and the estimation was continued according to Carlson & Wadström. The presence of methanol ensured a slightly higher degree of reproducibility. The overall recovery of 94% found by Carlson & Wadström was confirmed (*a*) by addition of known amounts of palmitic acid to sperm lipid and (*b*) by measuring the recovery of radioactivity from mixtures of tripalmitin and [^{14}C]palmitic acid (The Radiochemical Centre, Amersham, Bucks.) which had been subjected to two-stage chromatography.

Gas–liquid chromatography of free fatty acids. The methyl esters were separated at 170° in an Argon Chromatograph (W. G. Pye and Co. Ltd., Cambridge). The column was 120 cm. × 0·5 cm. diameter and contained polyvinyl acetate on Celite (1:5).

Estimation of phosphorus and plasmalogen in the lecithin–plasmalogen fraction obtained by paper chromatography of sperm lipids

Chromatography of sperm lipids was carried out on silicic acid-impregnated paper in di*iso*butyl ketone–acetic acid–water (8:5:1) essentially by the method of Marinetti & Stotz (1956). These authors ran their chromatograms at 25°. Although plasmalogen is more stable under such conditions than would be supposed from its rapid decomposition in aqueous acetic acid, some decomposition does occur at 25° since a forward streaming of free aldehyde from the plasmalogen spot can be detected after developed chromatograms have been treated with 2:4-dinitrophenylhydrazine. This decomposition is less marked at lower temperatures. In our metabolic experiments plasmalogen decomposes to give lysoplasmalogen, which is far more acid-labile than is plasmalogen (Hartree & Mann, 1960*b*). Thus, although at 0° the decomposition of plasmalogen is virtually abolished, that of lysoplasmalogen is not. Lysoplasmalogen has a lower R_F than plasmalogen and during chromatography at 0° the forward streaming of fatty aldehyde liberated from lysoplasmalogen will tend to overlap the plasmalogen spot and thus give rise to too high values for the plasmalogen content of the spot. In the present work temperatures of 10° and 14° were used. These result in insignificant decomposition of plasmalogen, but the aldehyde arising from lysoplasmalogen moves well beyond the plasmalogen spot by the end of the run.

The procedure was as described by Hartree & Mann (1960*b*) with the following modifications. Spots of each of the three lipid samples *A*, *B* and *C* (2–3 μg. of phosphorus) were placed 6 cm. from the end of a paper strip (Whatman no. 1), 10 cm. × 36 cm., and ascending runs were carried out in individual narrow cylindrical jars for 16 hr. The papers were allowed to dry for 1 hr. in a current of air. Aldehydogenic lipids were detected as follows. (All washings were in running tap water.) The papers were soaked in 15% (v/v) ethanol for 30 min. and dried in air for 1 hr. They were then placed for 30 min. in 2:4-dinitrophenylhydrazine (7·5 mM in 2N-HCl). The major spot (R_F 0·6–0·65) was identified as choline plasmalogen by comparison with the chromatographic behaviour of Pangborn ox-heart lecithin (Hartree & Mann, 1960*b*), which is a mixture of two components, lecithin and choline plasmalogen, with equal R_F values. The papers were freed from 2:4-dinitrophenylhydrazine by successive washings in water (1 min.), 2N-HCl (10 min.), water (1 min.), 2N-HCl (10 min.) and water (5 min.). While still moist the papers were examined in long-wave u.v. light from a mercury-vapour lamp. The major positive spots, which are those of lecithin–plasmalogen, were outlined in pencil and the papers were allowed to dry. For analysis the spots were cut out so that equal areas of paper were removed with each of the three spots on the chromatogram. Control pieces of equal area were taken from between the lecithin–plasmalogen spots. Extraction of phospholipids from the spots was carried out within 12 hr. of treatment with 2:4-dinitrophenylhydrazine.

For estimation of phosphorus the cut-out spots were placed in 16 mm. diam. test tubes and extracted three times with methanol (total of 7·5 ml.). The extracts were transferred to a second batch of tubes containing glass beads and evaporated at 100°. Phosphorus was deter-

mined according to Chen, Toribara & Warner (1956): if the blue colour was developed in a volume of 4 ml., 1 μg. of phosphorus gave rise to an extinction of approximately 0·2 at 820 mμ. A strict proportionality between extinction and phosphorus content was obtained within the range 0–2·5 μg. of phosphorus.

Plasmalogen in the cut-out spots was determined as follows. The spots and blanks were placed in test tubes with 2 ml. of diethylene glycol monomethyl ether (ethyl-digol). The tubes were sealed with Parafilm and stored overnight at 37°. Next day they were treated with 1 ml. of freshly prepared alkaline ethyl-digol [200 ml. of ethyl-digol + 1 ml. of 50% (w/w) KOH] and the extinction at 440 mμ (E_{440}) was measured against pure ethyl-digol after 6–7 min. The blanks were also measured against ethyl-digol. A calibration curve was prepared as follows. Known volumes (5–30 μl.) of a standardized chloroform solution of crystalline lysoplasmalogen (Hartree & Mann, 1960*b*) were spotted on silicic acid-impregnated paper, which was then treated with 2:4-dinitrophenylhydrazine and washed as described above. The spots (with blanks) were cut out and subjected to ethyl-digol extraction. A straight-line relationship was obtained between E_{440} and quantity of lysoplasmalogen. After correction for the blank reading (0·030) this relationship could be expressed as μequiv. of aldehyde = 0·141 E_{440} (S.E.M. of 15 estimations = 0·74%). A similar reproducibility was obtained when total lipids of spermatozoa were spotted quantitatively on paper. However, if chromatograms of sperm lipids were developed before the treatment with 2:4-dinitrophenylhydrazine (as in the analysis of lipids *A*, *B* and *C*) the results were naturally less reproducible (S.E.M. about 2–4%).

Other analytical procedures

Fructose was determined by the method of Roe (1934) as adapted to semen by Mann (1946), citric acid according to Speck, Moulder & Evans (1946), and lactic acid according to Barker & Summerson (1941). Fatty acyl ester and, except where otherwise stated, plasmalogen were estimated as by Hartree & Mann (1959). Choline was determined by Glick's (1944) method.

Nitrogen was estimated after incineration with H_2SO_4 and copper selenite. The method of Umbreit, Burris & Stauffer (1957) was used with the following modification, which ensured a straight-line relationship between nitrogen content (0–80 μg.) and colorimeter reading when a light-filter with maximum transmission at 520 mμ was used. The Nessler reagent was made up to contain twice the recommended concentration of gum ghatti and 3 ml. of reagent was used in place of 1·5 ml.

Bound amino acids were detected and approximately estimated by two-dimensional paper chromatography of an acid hydrolysate. The method used was that of Redfield (1953) as modified by Hunt (1959). The phospholipid (0·5 mg. of nitrogen) was heated in a sealed tube under nitrogen with 6 ml. of 6N-HCl for 15 hr. at 110°. The resulting aqueous phase was filtered through HCl-washed cotton wool and evaporated on a water bath. The residue was heated with 5 ml. of 0·2N-HCl plus 50 mg. of talc and filtered. The solution was repeatedly evaporated on the water bath with distilled water to remove HCl, the residue was dissolved in 0·2 ml. of water and samples were taken for chromatography.

Lipid solvents were purified as described by Hartree & Mann (1959). All evaporations were carried out under reduced pressure in a rotary evaporator.

Expression of analytical results

Wherever possible results are expressed in terms of m-equiv. present in the spermatozoa or plasma obtainable from 100 ml. of whole semen (m-equiv./100 ml. of semen). Corrections have been applied for the losses (up to 7% of cells) that occurred during the low-speed centrifugal washing of the spermatozoa used for the experiments in Table 3.

Rates of O_2 uptake are given as μl. of $O_2/10^8$ cells.

RESULTS

Examination of phospholipid fractions obtained from spermatozoa and seminal plasma by chromatography on silicic acid columns

The analyses of the various fractions obtained from ram spermatozoa and seminal plasma, with the exception of amino acid determinations, are listed in Table 1.

Phospholipids from freeze-dried spermatozoa. Phospholipid (29·1 mg. of phosphorus) equivalent to 18·8 g. of dry spermatozoa was applied to a 27 mm. diameter column, which was developed with 60 ml. of chloroform and then successively with the following chloroform–methanol mixtures: ·6:1 (350 ml.); 2:1 (500 ml.); 3:2 (250 ml.); 1:1 (400 ml.). The elution pattern, with three major peaks, is shown in Fig. 1.

The contents of the fraction-collector tubes were combined to give four main fractions: 1, tubes 20–29; 2, tubes 31–50; 3, tubes 83–100; 4, tubes 105–200.

Phospholipids from frozen spermatozoa and from frozen seminal plasma. Thawed, washed spermatozoa (50 ml.), originating from the same volume of semen, yielded total lipid containing 9·0 mg. of phosphorus. Of the phospholipids separated from this material, an amount containing 6·45 mg. of phosphorus was fractionated on a silicic acid column (20 mm. diameter) which was developed with chloroform–methanol (7:1) until no more phosphorus came through and then with chloroform–methanol (3:2) until no further phospholipid was eluted. The elution pattern exhibited three major peaks of which the last represented the major component (Fig. 2, continuous line). The collector tubes were grouped to yield three fractions: 1, tubes 9–11; 2, tubes 12–16; 3 (lecithin–plasmalogen fraction), tubes 39–65.

From thawed seminal plasma (80 ml.; equivalent to 100 ml. of semen) phospholipid containing 4·6 mg. of phosphorus was isolated. Chromatographic analysis (column diameter 14 mm.) gave an elution pattern similar to that of the sperm lipid.

Table 1. *Analysis of fractions obtained by chromatography on silicic acid columns of phospholipids extracted from ram spermatozoa and from seminal plasma*

Percentage of plasmalogen is derived from 200 × aldehyde/(aldehyde + ester) except in the last line where it is calculated from 100 × aldehyde/choline. LP, Lecithin–plasmalogen fraction (see Methods).

	Molar ratio (P = 1·00)				Phosphorus		Plasmalogen in LP fraction (%)
	Nitrogen	Choline	Aldehyde	Acyl ester	(m.equiv./100 ml. of semen)	(%)	
(*a*) Freeze-dried washed spermatozoa:							
Fraction 1	0·22	0	0·17	2·64	—	1·8	—
Fraction 2	1·02	0	0·80	1·96	—	3·0	—
Fraction 3*	1·08	0·80	1·18	1·44	—	3·2	—
Fraction 4*	1·37	0·94	0·93	0·97	—	2·9	98
(*b*) Frozen washed spermatozoa:							
Tubes 9–11	0·28	—	—	—	0·044	—	—
Tubes 12–16	0·88	—	—	—	0·047	—	—
LP fraction	0·98	0·98	0·81	1·22	0·275	4·0	80
(*c*) Frozen seminal plasma:							
LP fraction	—	0·98	0·83	1·23	0·057	—	81
Fresh washed spermatozoa:							
LP fraction	1·00	0·84	0·48	1·24	0·266	3·6	55
Fresh washed spermatozoa used for metabolic experiments:							
LP fraction*	1·10	0·83	0·40	1·23	—	3·7	49
Frozen whole semen, results obtained by Gray (1960*a*):							
Kephalin fraction	0·78	—	—	—	—	—	—
LP fraction	0·95	0·92	0·50	—	—	—	55

* Examined for amino acids (see Table 2).

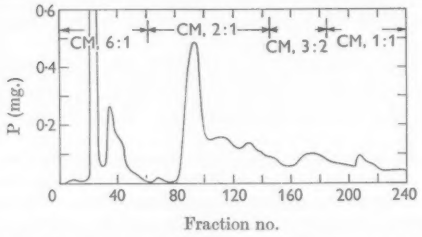

Fig. 1. Chromatography on a silicic acid column of phospholipids isolated from freeze-dried ram spermatozoa. Phospholipid from 18·8 g. of dried cells (29·1 mg. of P) was applied to a 27 mm. diameter column containing 2 g. of silicic acid and 1 g. of Hyflo SuperCel/mg. of P. The first peak reached a height corresponding to 1·58 mg. of P. CM, Chloroform–methanol, with ratios v/v.

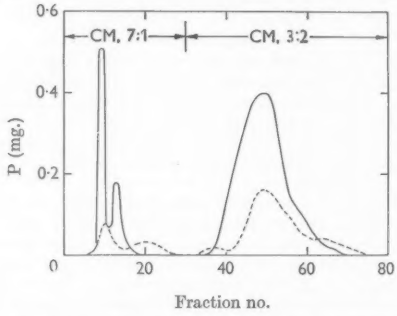

Fig. 2. Chromatography on silicic acid of phospholipids from frozen spermatozoa and frozen seminal plasma. —, Phospholipids from spermatozoa (9 mg. of P; 20 mm. diameter column); - - -, phospholipids from plasma (4·6 mg. of P; 14 mm. diameter column). Column weights were as in Fig. 1. CM, Chloroform–methanol, with ratios v/v.

An additional component appeared immediately before the lecithin–plasmalogen fraction, but the two were well separated (Fig. 2, broken line).

Phospholipids from fresh spermatozoa. Two batches of 20 ml. of fresh semen were washed (procedure 1) and made up to 1·5 vol. with sperm Ringer solution. When the extracted phospholipid (7·1 mg. of phosphorus) was subjected to column fractionation, lecithin–plasmalogen was eluted as a distinct fraction by chloroform–methanol (3:2).

Phospholipids from fresh spermatozoa that had been used for metabolic experiments. In experiments designed to show metabolic changes in the concentration of free fatty acids in spermatozoa (see below) washed suspensions were divided into three portions, of which two were incubated under anaerobic and aerobic conditions respectively,

while the third served as control. Lipids were extracted from the three portions and fractionated to yield free fatty acids. The first stage in the fractionation was the absorption of phospholipids on silicic acid columns. The phospholipids from five such experiments (15 portions) were dissolved from the columns with methanol, pooled and re-fractionated twice on larger columns. In the first run the successive solvents were chloroform and chloroform–methanol (4:1). The material extracted with the latter solvent was transferred to a second column, which was developed successively with chloroform–methanol (7:1 and 3:2). As in the previous runs, the lecithin–plasmalogen fraction was clearly separated during elution with the latter solvent.

Amino acids in lecithin–plasmalogen fractions. Three fractions (see Table 1) in which the N:P ratio exceeded 1·0 were hydrolysed with HCl and examined by paper chromatography (Table 2). In only one case was ninhydrin-positive material detected: thus fraction 3 from freeze-dried spermatozoa (Table 1, *a*) showed a single spot of phenylalanine. However, the spot intensity corresponded to $\nearrow$ 0·02 mole of phenylalanine/mole of phospholipid.

Changes in the lipid content of washed ram spermatozoa after periods of anaerobic and aerobic metabolism

Four experiments have been carried out for the purpose of comparing the free fatty acid and lecithin–plasmalogen contents of ram spermatozoa before and after periods of metabolic activity. These experiments were carried out within the period December 1959–March 1960, i.e. during the peak of the breeding season when motility and respiration of spermatozoa were consistently high. Details of incubation of the spermatozoa are given in Table 3, where *A*, *B* and *C* denote control, anaerobically and aerobically incubated spermatozoa respectively (see Methods). Of each of the three lipid samples of Expt. i, 20 % was set aside for phospholipid analysis by paper chromatography while the remainders were used for estimation of total fatty acid by the iron–hydroxamate method. In Expts. ii and iii the entire lipid extracts were used for fatty acid estimation. The lipid samples *A*, *B* and *C* of Expts. iv*a* and iv*b* were combined in pairs and 20 % of each was set aside for phospholipid analysis. The fatty acid samples

Table 2. *Amino acids in hydrolysates of the plasmalogen-rich phospholipid fractions having* N:P *ratios* > 1·00

The amino acids were separated by two-dimensional paper chromatography. After the papers had been sprayed with ninhydrin, phenylalanine was estimated approximately by comparison with standard spots. LP, Lecithin–plasmalogen fraction (see Methods).

Phospholipid fraction (see Table 1)	Phospholipid equivalent of hydrolysate applied to paper (μg.atoms of P)	Amino acid detected	Ratio moles of amino acid/g.atoms of P
Expt. *a*, fraction 3	11·1	~0·2 μmole of phenylalanine	0·02
Expt. *a*, fraction 4	16·4	None	< 0·0003
LP fraction of spermatozoa from metabolic experiments	27·4	None	< 0·0002

Table 3. *Changes in free fatty acid contents of suspensions of washed spermatozoa resulting from periods of anaerobic and aerobic metabolism at* 37°

Each experiment involved three 15 ml. samples (*A*, *B* and *C*) of a suspension of spermatozoa in sperm Ringer solution. Control samples (*A*) were treated with chloroform–methanol immediately after the suspension had been prepared. Anaerobic (*B*) conditions were achieved by storing the suspensions in stoppered tubes with minimum air space. Aerobic (*C*) incubations were carried out in Barcroft differential manometers. Fatty acids were isolated by two-stage column chromatography and estimated by the iron–hydroxamate method. (For further details see text.)

Expt. no.	10^{-8} × Sperm density (cells/ml.)	Incubation time (min.)	Total O_2 uptake (μl./10^8 cells)	Total O_2 uptake (m-moles/100 ml. of semen)	Free fatty acid found (m-equiv./100 ml. of semen) *A*	*B*	*C*	O_2 uptake/fatty acid oxidized (molar ratio)
i	13·0	155	19·5	2·65	0·442	0·629	0·366	10·0
ii	13·8	175	15·7	2·31	0·349	0·723	0·525	11·7
iii	9·7	175	20·2	2·95	0·343	0·470	0·165	9·7
iv*a*	10·4	175	15·7	3·38	0·298	0·492	0·180	10·8
b	10·4	180	26·9					

obtained from the remaining lipids of iv $(a+b)$ were each divided into two equal parts, one being used for determinations of total fatty acid and the other for gas–liquid chromatography.

Fatty acids. Analyses of total free fatty acid in the *A*, *B* and *C* samples (Table 3) show that there is a distinct accumulation of acids during periods of anaerobic metabolism, whereas during aerobic metabolism there is either a smaller accumulation (Expt. ii) or a net loss.

Gas–liquid chromatography of fatty acids in samples *A*, *B* and *C* (Table 4) showed that it was mainly the C_{14} and C_{16} acids which accumulated during anaerobic incubation and that these same acids were preferentially oxidized if oxygen was present. Gray (1960*b*) has analysed the fatty acids liberated by hydrolysis of choline plasmalogen in whole ram semen. The major components of the mixture (C atoms: no. of double bonds) were as follows (figures in parentheses indicate percentage of each component in the recovered acids): 14:0 (5·8), 16:0 (32·0), 16:1 (5·4), 17:1 (10·1), 18:0 (11·7): 18:1 (9·5), 20:4 (11·5). By comparison, our Table 4 shows (1) a similar ratio for the C_{14} and C_{16} acids, (2) no C_{17} acid, (3) larger quantities of C_{18} acids. There is no reason why Gray's results should be similar to ours since the fatty acids isolated by us are not necessarily derived completely from plasmalogen. Further, the plasmalogen that was hydrolysed in metabolic experiments was no doubt localized in regions of high metabolic activity and it cannot be assumed that such plasmalogens would be representative, in terms of fatty acid composition, of the semen plasmalogen as a whole.

Phosphorus and plasmalogen. Samples of sperm phospholipid from Expts. i and iv (Table 3) were subjected to chromatography on silicic acid-impregnated papers, each of which carried equivalent amounts of lipids *A*, *B* and *C*. Four identical papers were run at one time and the spots identified after the treatment with 2:4-dinitrophenylhydrazine. The six lecithin–plasmalogen spots from two of the papers were analysed for phosphorus and the remaining six from the other two papers for plasmalogen. Replicate runs were made in order that the S.E.M. could be calculated from 12–16 papers for both phosphorus and plasmalogen. Spots that were adequate for analysis, and at the same time clearly separated from other lipid components, were obtained when the amounts of lipid placed upon the paper were such as to give lecithin–plasmalogen spots containing 0·01–0·02 μequiv. of plasmalogen.

Analyses for phosphorus and plasmalogen are summarized in Table 5. In the first column are

Table 4. *Gas–liquid chromatography of free fatty acids isolated from sperm lipids*

The acids were converted into methyl esters and run at 170° on polyvinyl acetate–Celite (1:5). The shortened designations are those used by Ahrens *et al.* (1959). Thus 16:1 represents a monounsaturated C_{16} acid. *A*, Control; *B*, anaerobic; *C*, aerobic.

Sample from Expt. iv (Table 3)	*A*		*B*		*C*	
Fatty acid (shortened designation)	(% of total)	(m-equiv./100 ml. of semen)	(% of total)	(m-equiv./100 ml. of semen)	(% of total)	(m-equiv./100 ml. of semen)
14:0	5·7	0·02	34·4	0·17	15·6	0·02
16:0	29·8	0·09	33·0	0·16	44·8	0·07
16:1	3·9	0·01	2·2	0·01	3·8	0·01
18:0	22·8	0·07	12·9	0·06	8·1	0·01
18:1	30·8	0·09	11·4	0·06	19·2	0·03
18:2	7·0	0·02	6·2	0·03	8·6	0·01

Table 5. *Phosphorus and plasmalogen contents of the lecithin–plasmalogen spots from paper chromatograms of lipid samples* A, B *and* C

The samples were obtained from the lipids isolated during metabolic Expts. i and iv of Table 3. The S.E.M. was calculated in all cases from between 12 and 16 replicate chromatograms. *A*, Control; *B*, anaerobic; *C*, aerobic.

Expt. no. (see Table 3)		Analysis of samples *A* (μequiv./ml. of lipid applied to the paper)	Equivalents present in lecithin–plasmalogen spots relative to *A* = 100		
			A	*B*	*C*
i	Phosphorus	3·95±0·198	—	—	—
	Plasmalogen	1·86±0·082	(100)	76·9±4·20	79·6±3·77
iv	Phosphorus	2·33±0·186	(100)	89·1±4·10	88·8±4·46
	Plasmalogen	1·11±0·078	(100)	74·5±4·25	72·0±3·04

given the mean values for phosphorus and plasmalogen in the lecithin–plasmalogen spots derived from lipids *A*. For each chromatogram the phosphorus and plasmalogen present in lipids *B* and *C* were calculated as percentages of the corresponding values for lipid *A*. The means of these percentages are shown in the last two columns.

Effect of various fractions prepared from semen on the metabolism of washed ram spermatozoa

After ram spermatozoa have been washed by procedure 1 respiratory activity is initially equal to and sometimes slightly greater than that of whole semen, but the rate of oxygen consumption decreases considerably with time and a fall is already noticeable after aerobic incubation for ½ hr. at 37°. The addition of fructose to such suspensions causes the initial rate of oxygen consumption to be restored to that of whole semen, and the high level to be maintained for several hours (Hartree & Mann, 1960*a*). On the other hand, after wash procedure 2, restoration of respiratory activity by fructose is far from complete and motility is also impaired. For this reason the following experiments on activation of sperm respiration were performed with spermatozoa which had been washed by the milder procedure 1. The seminal fractions used for the purpose of activation were, however, obtained from semen fractionated by procedure 2. The latter procedure gives more complete separation of spermatozoa from plasma; further, the wash fluid obtained by this procedure, unlike that obtained by procedure 1, consists not merely of diluted plasma but also contains material that, under the influence of high centrifugal speeds, has been removed from the outer layers of the sperm cells. The efficiency of the washing process can be judged from the following analyses of citric acid, which is a normal constituent of ram seminal plasma. Thus separation according to procedure 1 gave spermatozoa, seminal plasma and wash fluid containing respectively < 4, 352 and 15 mg. of citric acid/100 ml. of semen. After procedure 2, the figure for spermatozoa was 13. Citric acid, unlike fructose, is not metabolized by spermatozoa (Humphrey & Mann, 1949) and thus serves as a more reliable indicator of the degree of separation of plasma.

The effects of a dialysed concentrate of wash fluid and of dialysed seminal plasma on the respiration of washed spermatozoa are illustrated in Fig. 3. The residual fructose present in these two fractions contributed only 8 and 12 μg. of fructose respectively to the manometer flasks. These quantities of fructose would have negligible effects upon the respiration of washed spermatozoa: in fact they would be completely metabolized during the 10 min. equilibration period preceding closure of the manometer taps. The magnitude of the accelerating effect of the two fractions decreases with increasing sperm density. Thus Fig. 3 illustrates experiments in which the sperm density was $4{\cdot}1 \times 10^8$ cells/ml. In a parallel experiment with a density of $6{\cdot}4 \times 10^8$ the rate of respiration in the control was 50% higher but the activating effects of wash fluid and seminal plasma were smaller. Such inverse relationships between sperm density and activation by semen fractions were commonly observed.

Fig. 4 gives the results of experiments with a sample of washed spermatozoa of abnormally low respiratory activity. The addition of fructose to this sample raised the respiratory activity to the level usually observed with normal spermatozoa in the presence of fructose (cf. Figs. 3 and 4). Respiration was also increased significantly by a lecithin–plasmalogen fraction (Table 1, *b*) which had been purified by column chromatography. However, this same fraction usually had very little and sometimes no activating effect upon the respiration of normal spermatozoa and we were unable to find evidence that it could be metabolized by washed spermatozoa (see below).

The accelerating effect of 'total lipid' obtained from the concentrate of wash fluid was traced to the presence of diffusible metabolites. Thus when the

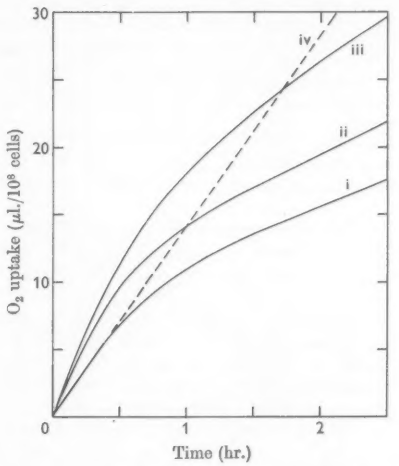

Fig. 3. Influence upon the respiratory activity of washed spermatozoa of dialysed seminal plasma, of dialysed concentrate of wash fluid and of fructose. To each manometer was added a suspension of $12{\cdot}3 \times 10^8$ cells in 2 ml. of sperm Ringer solution. Volumes were made up to 3 ml. as follows: i, 1 ml. of sperm Ringer solution (control); ii, 1 ml. of dialysed seminal plasma; iii, 1 ml. of dialysed concentrate of wash fluid (≡ washings from spermatozoa in 7·7 ml. of semen); iv, 1 ml. of 10 mM-fructose in sperm Ringer solution. Temp. 37°.

lipid was obtained from a concentrate that had been exhaustively dialysed it had no accelerating effect upon respiratory activity (Fig. 5). This 'dialysed total lipid' contained approximately 0·1 % of lactic acid and negligible fructose, whereas 'total lipid' (from non-dialysed concentrate) contained 2·9 % of lactic acid and 0·22 % of fructose, despite the washing of the chloroform solution of lipid with magnesium chloride solution (Folch *et al.* 1957). The 'free fatty acids' of ram spermatozoa brought about a barely significant acceleration of sperm respiration (Fig. 5) and the 'non-lipid, non-diffusible fraction' had no effect.

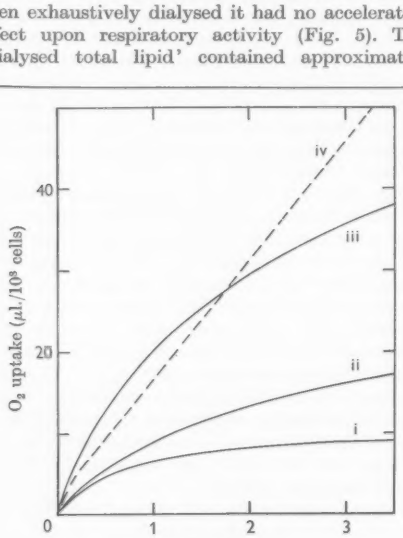

Fig. 4. Effects on the respiration of washed spermatozoa of a lecithin–plasmalogen fraction derived from spermatozoa, of total lipid extracted from wash fluids and of fructose. Each manometer received $9{\cdot}7 \times 10^8$ spermatozoa; otherwise the conditions were as for Fig. 1. i, Control; ii, with 10·2 mg. of lecithin–plasmalogen (Table 1, *b*); iii, with 9·8 mg. of total lipid from wash fluid; iv, with 10 mM-fructose.

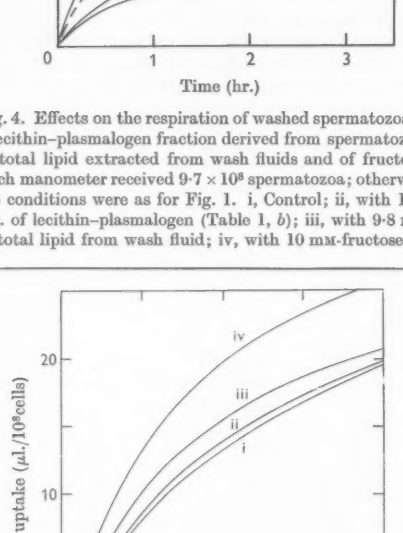

Fig. 5. Effects of sperm lipid fractions upon the respiration of washed spermatozoa. Each manometer received $16{\cdot}4 \times 10^8$ spermatozoa; otherwise the conditions were as for Fig. 1. i, Control; ii, with 11·4 mg. of dialysed total lipid from wash fluid; iii, with 6·1 mg. of free fatty acids from spermatozoa; iv, with 9·8 mg. of total lipid from wash fluid.

Inability of washed spermatozoa to metabolize added lecithin–plasmalogen extracted from spermatozoa

A suspension of washed ram spermatozoa in sperm Ringer solution (wash procedure 1) containing $4{\cdot}9 \times 10^8$ cells/ml. was divided into two parts. To one (S+) was added the lecithin–plasmalogen fraction from ram spermatozoa (Table 1, *b*) at a concentration of 3·4 mg./ml. The other part (S−) received no additions. Each part was divided into three samples, which were treated as the samples *A*, *B* and *C* described above, the incubation time being 135 min. Lipid was extracted from the six samples and analysed for acyl ester and plasmalogen. In Table 6 are shown the excess of acyl ester and of plasmalogen in the S+ samples above the levels in the corresponding S− samples. It is clear that no appreciable metabolism of added lecithin or plasmalogen occurred.

DISCUSSION

Effects of added lipids on spermatozoa

In view of the long-entrenched belief that sperm lipids are chiefly composed of lecithin, it is not surprising that experiments on the effect of added phospholipids on sperm motility and metabolism have hitherto been confined to lecithin, mostly in the form of preparations obtained from egg-yolk (Lardy & Phillips, 1941*b*; Bomstein & Steberl,

Table 6. *Inability of washed ram spermatozoa to metabolize added lecithin–plasmalogen isolated from spermatozoa*

S+, A suspension of spermatozoa ($4{\cdot}9 \times 10^8$ cells/ml.) in Ringer solution containing 3·4 mg. of lecithin–plasmalogen/ml. (Table 1, *b*); S−, the same suspension without lecithin–plasmalogen. The two suspensions were each divided into three parts and treated as samples *A*, *B* and *C* (Table 3). Incubation was for 135 min. at 37°. The lipids extracted from the six samples were analysed for acyl ester and for plasmalogen. *A*, Control; *B*, anaerobic; *C*, aerobic.

	Excess in S+ over S− suspensions (μequiv./ml.)		
	A	*B*	*C*
Acyl ester	3·78	3·88	3·78
Plasmalogen	2·48	2·62	2·53

1957). Recent research, however, has revealed the presence of a high concentration of choline-based plasmalogen in spermatozoa and, at the same time, has provided a strong indication that this aldehydogenic phospholipid is involved in aerobic sperm metabolism (Hartree & Mann, 1959, 1960*a*). We have therefore extracted from ram spermatozoa both the total phospholipid as well as the lecithin–plasmalogen fraction and examined the effect of these preparations on the aerobic metabolism of washed ram spermatozoa. Crude total phospholipid had usually quite a strong stimulating effect on sperm respiration, but this was shown to be due, not to lipids as such, but to impurities such as fructose and lactic acid which are carried over into such lipid preparations during the extraction procedure. The lecithin–plasmalogen fraction on the other hand separated by the use of silicic acid columns, had only occasionally a small stimulating influence on sperm respiration, whereas in most instances, particularly when sufficient care was taken to protect the spermatozoa from loss of motility and metabolic activity during washing, no effect or only a negligible rise was observed. Marked activating effects on sperm respiration were, however, observed with dialysed concentrates of wash-fluid and with dialysed seminal plasma, but these were probably due to a protective action on the sperm cells of either lipoproteins or some other protein constituents. Similar effects, usually attributed to the stabilizing action of 'protective colloids', are believed to account for the success of many semen diluents, including the egg-yolk buffer diluent, in the preservation of semen for the purpose of artificial insemination (Phillips, 1939; Phillips & Lardy, 1940; Mann, 1954).

Composition of choline-containing lipids extracted from spermatozoa and seminal plasma

The composition of the phospholipids extracted from ram spermatozoa appears to vary according to the treatment to which the cells were subjected before the actual extraction with lipid solvents. This difference becomes particularly obvious when the results from analysis of fresh or wet-frozen spermatozoa are compared with those obtained with freeze-dried material, and it may well account for some of the conflicting views expressed by Lovern *et al.* (1957) and Gray (1960*a*).

With sperm material which had not been subjected to freeze-drying before extraction, the elution of lipids from silicic acid columns follows the usual pattern (Hanahan *et al.* 1957). The first fraction to emerge has a low N:P ratio, and presumably contains the polyglycerol phosphatide which has been characterized by McKibbin & Taylor (1952). This is followed closely by the kephalin fraction. A solvent richer in methanol then elutes the major fraction in which most, or all, of the nitrogen is due to choline, and which we have named the lecithin–plasmalogen fraction since it contains both lecithin and choline-based plasmalogen. Finally, by the use of pure methanol, another fraction containing sphingolipids and lyso compounds can be eluted. This latter fraction is very small except for lipids extracted from washed spermatozoa that had been actively respiring in the absence of sugar for several hours at 37°.

With the lipids of freeze-dried sperm, the elution pattern is different in respect to the lecithin–plasmalogen fraction which, in effect, divides during chromatography into two fractions. While the first of these (no. 3) is eluted rapidly, the second (no. 4) follows slowly, and is responsible for the long 'tailing' of the plasmalogen fraction first described by Lovern *et al.* (1957). Moreover, when the solvents were removed, both fractions appeared as oils, in contrast with the waxy consistency of the lecithin–plasmalogen fraction obtained from non-dried sperm. The first of the two fractions mentioned above (no. 3) also exhibited some other unusual characteristics. Its content of fatty aldehyde + acyl ester was markedly in excess of phosphorus, probably owing to the presence of glycerides, and it contained some phenylalanine, possibly in the form of a lipoamino acid. The second fraction (no. 4) corresponded in composition to a mixture of 72% of plasmalogen and 28% of some material containing 1·8% of non-choline nitrogen. The ratio between acyl ester, aldehyde and choline was 1:1:1, which confirms the earlier findings of Lovern *et al.* (1957) that the lecithin–plasmalogen obtained from freeze-dried sperm consists mainly of plasmalogen and contains no appreciable quantity of lecithin.

As regards the nitrogen:choline ratio in lecithin–plasmalogen preparations, this tends to be higher than 1, whether or not the sperm had been freeze-dried before extraction. The nature of the excess of nitrogen is at present unknown. It is not due to amino acids, at least in fresh spermatozoa. The presence in phospholipid fractions of excessive nitrogen which could not be accounted for in terms of known bases or amino acids has also been noted by other investigators (McKibbin & Taylor, 1952; McKibbin, 1957).

Although it is clear from our study that the choline phosphatide fraction as isolated from non-dried material contains lecithin in addition to plasmalogen, there remains a disagreement between the results which Gray (1960*a*) obtained with frozen, whole ram semen, and our data, which are based on analyses of frozen washed spermatozoa and frozen seminal plasma (Table 1). The lecithin–plasmalogen fraction that we prepared from washed spermatozoa and seminal plasma contained to-

gether 232 μequiv. of choline/100 ml. of semen. Only 12·6 μequiv. of choline/100 ml. of semen were present in the wash-fluid from spermatozoa, and even this small quantity was probably due mostly to glycerylphosphorylcholine rather than to lipids. Of the lipid contained in the lecithin–plasmalogen fractions of frozen spermatozoa and frozen plasma, 80 % was in the form of plasmalogen as compared with 55 % reported by Gray (1960*a*). It seems unlikely that this difference can only be due to the fact that whereas Gray used whole semen, we performed our analyses after the semen had been separated into sperm and seminal plasma. A more likely explanation may lie in different storage conditions, particularly with regard to the actual temperature and duration of freezing. It is quite possible that under certain cold-storage conditions, sperm lecithin is rendered non-extractable. This possibility is borne out strongly by our analyses of a lecithin–plasmalogen fraction prepared from freshly washed, non-frozen ram spermatozoa. In such material the plasmalogen/lecithin ratio was, in fact, nearly 1:1 and very close to the value reported by Gray (1960*a*). It would appear from our study that fresh, rather than frozen, material should if possible be used for studies on phospholipid distribution.

Breakdown of plasmalogen in washed spermatozoa

We previously suggested (Hartree & Mann, 1959) that in the absence of seminal fructose the energy requirements of washed spermatozoa are derived from the oxidation of fatty acids and that these acids arise through the breakdown of plasmalogen. This view was of necessity based upon the belief that lecithin is not present in ram spermatozoa, since our previous methods could not distinguish between lecithin and plasmalogen as the source of fatty acid. We have found (Hartree & Mann, 1959) that, in sugar-free suspensions, the rate of hydrolysis of fatty acyl ester in washed ram spermatozoa is the same in the presence as in the absence of oxygen. The experiments reported in the present study, however, show that the net accumulation of fatty acids in such suspensions is always lower after aerobic than after anaerobic incubation. Thus, in the presence of oxygen, fatty acids are metabolized and such metabolic activity, expressed as the difference between anaerobic and aerobic levels, is proportional to activity in terms of oxygen utilization (Table 3). Chromatographic analysis of the fatty acids indicates that the rise in the fatty acid concentration which one observes in anaerobically incubated sperm samples is largely accounted for by an increase in the content of myristic acid and palmitic acid and that the aerobic disappearance is also mainly due to the same two acids. Our previous calculations (Hartree & Mann, 1959) on the ratio between O_2 uptake and fatty acids oxidized were based on an assumption that the mean length of these fatty acids was about 20 carbon atoms. However, even if oxidations in spermatozoa should involve only C_{14} and C_{16} fatty acids in approximately equimolar amounts, such a process would still require 22 mol.prop. of oxygen for complete combustion. Our results (Table 3, final column) show that the actual amounts of oxygen consumed by washed spermatozoa are consistently below this value, and support the view that fatty acids are available in excess of the amount theoretically required to support respiratory activity. In addition, there is the possibility that the incubation mixture may also contain other utilizable fatty acids, of chain length C_{12} or less, which would not be detected by our method of analysis.

The results given in Table 5 confirm that the ratio lecithin:choline plasmalogen in freshly washed spermatozoa is close to unity; and in fact the analysis of the control sample of Expt. iv gives a value of 48 % of plasmalogen. In the same experiment the decreases in lecithin–plasmalogen phosphorus during anaerobic and aerobic incubation were 10·9 and 11·2 % respectively. If these decreases were due entirely to disappearance of plasmalogen the decreases in the latter would have been 23·6 and 24·3 % respectively. The agreement between these figures and the experimentally determined decreases in plasmalogen (Table 5, last line) leads to the conclusion that, within experimental error, there was no net breakdown of lecithin during our incubation experiments and that the sperm plasmalogen was the source of the fatty acids that appeared during anaerobic incubation. Such metabolic experiments do not rule out the possibility of a turnover of lecithin as envisaged by Dawson (1957). They also give a figure for percentage of plasmalogen in the lecithin–plasmalogen fraction which will be lower than the value *in vivo* since some decomposition will occur during the interval between separation of sperm from plasma and the beginning of a metabolic experiment. A similar low value (49 %) was obtained from the analysis of the lecithin–plasmalogen fraction of spermatozoa from other metabolic experiments (Table 1). Thus the value 55 % (Table 1) is probably more representative of ram spermatozoa *in vivo*.

The demonstration that spermatozoa can utilize their intracellular plasmalogen as a source of energy raises the question of the physiological importance of this lipid for sperm motility and survival, in comparison with extracellular substrates which are present in semen, such as fructose and lactic acid. Fructose has the advantage over both lactic acid and plasmalogen of being able to serve as a substrate not only aerobically but in the

absence of oxygen as well. Its value would therefore be greatest when spermatozoa are present in a highly concentrated state, such as may exist for example in freshly ejaculated semen, at the site of deposition in the female reproductive tract. An average ram ejaculate of 1·2 ml., however, with 4×10^9 spermatozoa, 4 mg. of fructose, and a fructolysis index of 2 mg. of fructose/10^9 sperm/hr. at 37° (Mann, 1948), would have converted anaerobically all its fructose into lactic acid within about 15 min., and after that sperm survival would not be possible without an alternative source of energy. But by that time the process of sperm passage through the female reproductive tract has already begun, and it is presumably at that stage when the ability to oxidize extracellular lactic acid or intracellular plasmalogen assumes the greatest importance to spermatozoa. Provided that the partial pressure of oxygen within the uterus does not fall below that which is characteristic of animal tissues in general, there is no reason to suppose that the spermatozoa would be prevented from using these oxidizable substrates. It is interesting to recall, in this connexion, the observation that the rate of oxygen uptake of ram spermatozoa remains practically unaltered when the oxygen concentration has been lowered from 100 to 4% (Humphrey & Mann, 1949). In addition, it has to be remembered that the energy which a cell can derive from the aerobic breakdown of a carbohydrate or a fatty acid is greater by far than that obtainable from glycolysis alone. Thus, as recently pointed out by Rothschild (1960), the complete oxidation of fructose can provide the sperm cell with about 24 times as much energy as can anaerobic fructolysis. All this, together with the long-established fact that many of the ram spermatozoa which reach the Fallopian tubes are still perfectly motile (Quinlan, Maré & Roux, 1932), suggests that sperm cells should have no difficulty in maintaining their energy requirements *in vivo* for as long as they can depend either on extracellular oxidizable substrates such as lactic acid, of which there is an abundance both in the semen as well as in the secretions of the female tract, or on intracellular plasmalogen acting as a source of oxidizable fatty acids.

SUMMARY

1. The effects of lipid-containing fractions obtained from ram semen upon the respiration of ram spermatozoa have been examined.

2. Marked activations were observed with dialysed seminal plasma and dialysed concentrates of fluids used to wash the spermatozoa. These are ascribed to the presence of lipoproteins.

3. Phospholipid fractions sometimes activated sperm respiration to a small extent. However, activation was insignificant when care was taken to prevent impairment of motility during preliminary washing of the spermatozoa.

4. The phospholipids extractable by chloroform–methanol from washed spermatozoa and from seminal plasma have been fractionated on silicic acid columns.

5. We have confirmed our earlier finding that the major phospholipid in extracts of freeze-dried spermatozoa is choline plasmalogen and that lecithin is absent.

6. Extracts of fresh spermatozoa, and of spermatozoa and plasma which have been frozen, contain both plasmalogen and lecithin. For fresh spermatozoa the ratio is approximately 1:1.

7. The lipids extractable from spermatozoa that had been incubated in the absence of sugar under both anaerobic and aerobic conditions have been compared with those from non-incubated spermatozoa. Analyses were made of (*a*) free fatty acids, (*b*) choline plasmalogen, (*c*) phosphorus in the mixed lecithin–plasmalogen fraction.

8. Free fatty acids accumulated during anaerobic incubation, but in the presence of oxygen the accumulation was smaller or there was even a net loss. It was mainly C_{14} and C_{16} acids which accumulated in the absence of oxygen and that were oxidized in its presence.

9. There was no appreciable decrease in lecithin during incubation for 2·5–3 hr. at 37°, but about 25% of the intracellular plasmalogen was hydrolysed.

10. These results strengthen our earlier view that, in the absence of fructose, ram spermatozoa can utilize as a source of energy the fatty acids which derive from breakdown of plasmalogen.

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Condensed Tannins

11. ISOLATION OF A CONDENSED TANNIN FROM BLACK-WATTLE HEARTWOOD, AND SYNTHESIS OF (±)-7:3′:4′-TRIHYDROXYFLAVAN-4-OL*

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Bate-Smith & Swain (1953) suggested that leuco-anthocyanins, which are closely related to the catechins, should be regarded as prototypes of condensed tannins. In this connexion, Hillis (1954, 1956, 1958) has investigated the extent to which leuco-anthocyanins might be precursors of condensed tannins, and might be responsible for the red colour which develops when leather that has been tanned with mangrove and various *Eucalyptus* extracts is exposed to sunlight.

Robinson & Robinson (1935) isolated the first crystalline compound related to the flavan-3:4-diols, peltogynol, and thereafter King & Bottomley (1954) isolated the first member of the structurally simpler and more general series of crystalline flavan-3:4-diols. The progress of condensation of such compounds in the wood was examined (Roux & Evelyn, 1958*a*, *b*, 1960) by anthocyanidin formation, by number-average molecular weights and by tannin analyses on radial and vertical drillings. These methods give some evidence for the presence of intact flavan-3:4-diol units in polyleuco-anthocyanins, which may be regarded as one class of condensed tannins. The association of authenticated monomeric flavan-3:4-diols and the corresponding polyleuco-anthocyanin tannins, which Roux (1958*a*, *b*) demonstrated for the heartwoods of certain *Acacia* and *Schinopsis* spp., is similar to the association of leuco-anthocyanins and complex leuco-anthocyanins in cacao beans (Forsyth, 1952, 1955). On account of the isolation and chemical degradation of a complex leuco-cyanidin from cacao (Forsyth, 1953; Forsyth & Roberts, 1960), the isolation of other polyleuco-anthocyanins that may have tanning properties is of scientific importance. The isolation of one of these polymeric tannins from *A. mearnsii* is described, and certain of its properties are compared with those of synthetic (±)-7:3′:4′-trihydroxyflavan-4-ol.

* Part 10: Roux & Paulus (1961*c*).

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Analysis of C, H, acetyl groups, methoxyl groups and ash are by Weiler and Strauss, Oxford, and by K. Jones, Microanalytical Section,

National Chemical Research Laboratory, C.S.I.R., Pretoria. Infrared comparisons are by Dr J. R. Nunn of the same laboratories. Molecular weights are by A. E. Maihs of these laboratories. A 2dm. tube was used for optical rotations. Two-way chromatograms were developed in water-saturated butan-1-ol followed by 2% (v/v) acetic acid (solvent combination 1), or in water-saturated butan-2-ol followed by 2% (v/v) acetic acid (solvent combination 2).

Isolation of a (−)-leuco-fisetinidin tannin from the heartwood of Acacia mearnsii

Separation of the tannin. In addition to flavonoid compounds possessing high R_F values (Roux & Paulus, 1960, 1961*a*, *c*), the heartwoods of freshly felled trees contain a prominent component in the middle R_F range (0·47 and 0·25 in water-saturated butan-1-ol and 2%, v/v, acetic acid respectively). This compound showed tanning properties. The dried, wax-free ethyl acetate extract (75 g.) of black-wattle heartwood obtained as described by Roux & Paulus (1960) was added slowly with shaking to 400 ml. of the lower phase of a water–butan-2-ol–petroleum (b.p. 45–60°) (5:3:2) mixture. The solution was warmed to 60°, then cooled to room temperature and filtered. The filtrate was introduced into the first eight tubes of a 160 tube, 50 ml. underphase, automatic Craig countercurrent machine, and the mixture separated by 160 transfers as described by Roux & Paulus (1960). The tannin component was distributed in tubes 31–61, but in tubes 31–40 tannin of lower R_F value was present. The contents of tubes 41–61 were combined, the organic phase was separated, and the aqueous phase extracted with ethyl acetate. The combined organic phase and ethyl acetate extracts were reduced to small volume (200 ml.) under vacuum, and applied as bands to 40 sheets of Whatman no. 3 chromatographic paper (22½ in. × 18¼ in.). Chromatograms were developed by upward migration of 2% (v/v) acetic acid. The tannin band was located with toluene-*p*-sulphonic acid (Roux, 1957*b*) and ammoniacal silver nitrate spray reagents on test strips. Bands were removed and eluted with 70% (v/v) ethanol. The solid (0·7 g.) obtained on evaporation was pale brown.

Two-way paper chromatography showed traces of impurities accompanying the tannin. These were separated on paper chromatograms as above with butan-1-ol–acetic acid–water (6:1:2) (downward) followed by 2% (v/v) acetic acid (upward) for successive separations on different sheets. The buff-coloured product (0·4 g.) appeared free of associated compounds by two-way paper chromatography with solvent combinations 1 and 2.

The tannin settled from aqueous solution as a white sludge and did not crystallize from dilute ethanol, methanol or acetic acid. The tannin migrated as a discrete spot in 2% (v/v) acetic acid and in water-saturated butan-2-ol. In water-saturated butan-1-ol and butan-1-ol–acetic acid–water (4:1:5, upper phase) the tannin moved as an elongated oval spot, although it had been twice separated on paper sheets in butan-1-ol–acetic acid–water (6:1:2). The elongated oval spot was coloured uniformly when sprayed with ammoniacal silver nitrate (grey), bisdiazotized benzidine (yellow), toluene-*p*-sulphonic acid (pink) and ferric alum (green) reagents.

The tannin gave an immediate precipitate with the gelatin–salt reagent (Atkin & Thompson, 1939; Thomas & Frieden, 1923), indicating strong tanning properties. The monomeric (+)-7:3′:4′-trihydroxyflavan-3:4-diol from wattle heartwood gives no precipitate.

The tannin, dried at 110° for 2 hr., had $[\alpha]_D^{20} - 32{\cdot}8 \pm 0{\cdot}4°$ in methanol (*c*, 0·8), and $-33{\cdot}3 \pm 0{\cdot}5°$ in acetone–water (1:1) (*c*, 0·6); $\lambda_{max.}$ 205 and 280·5 mμ in water and 283 mμ in ethanol; $E_{1cm.}^{1\%}$ 190·8, 193·1 in ethanol [$E_{1cm.}^{1\%}$ for (+)-mollisacacidin, 223·7, 222·9] (Found: C, 61·3, 61·2, 62·2; H, 5·4, 5·4, 5·8; ash, 0·0. $C_{15}H_{14}O_6$ requires C, 62·1; H, 4·9%). The infrared-absorption curve over the range 2·5–15 μ was similar to that of (+)-7:3′:4′-trihydroxyflavan-3:4-diol, and showed the absence of significant carbonyl-group absorption.

The tannin gave resorcinol, β-resorcylic acid and protocatechuic acid on microfusion with KOH under anhydrous conditions (Roux, 1958*c*). Traces of phloroglucinol (purple with bisdiazotized benzidine) were also detected, but other resorcinol-containing flavans, e.g. (−)-robinetinidol and (+)-7:3′:4′-trihydroxyflavan-3:4-diol also yield traces of phloroglucinol. The colour reactions of the tannin with the spray reagents was consistent with the presence of resorcinol and catechol nuclei only, and the pink with toluene-*p*-sulphonic acid suggested a 3:4-diol grouping (cf. Roux & Maihs, 1960). A test for *p*-hydroxybenzyl alcohol groups with quinonemonochloroimide (Gierer, 1954) was inconclusive.

A deep-pink colour was produced when the tannin (1 mg.) was heated in 3N-HCl (1 ml.) and propan-2-ol (4 ml.) solution under pressure at 100° (Pigman, Anderson, Fischer, Buchanan & Browning, 1953). The pink was shown to be due entirely to fisetinidin chloride, by paper-chromatographic comparison with synthetic material (Leon & Robinson, 1931; Freudenberg & Maitland, 1934); R_F 0·43 in 90% formic acid–3N-HCl (1:1) and 0·73 in water–acetic acid–10N-HCl (10:30:3); $\lambda_{max.}$ 523 mμ shifted to 540 mμ (blue) after forming a complex with $Al_2(SO_4)_3$ (cf. Roux, 1957*a*). Both the tannin and (+)-7:3′:4′-trihydroxyflavan-3:4-diol gave increasing yields of fisetinidin up to 90 min. (maximum yield) under the above conditions, the concentration of fisetinidin declining gradually if the tubes were heated for longer periods. From $\epsilon_{max.}$ values of fisetinidin chloride calculated from data of Roux & Evelyn (1958*a*) the yield from the tannin was 6·9–7·3%, compared with 22·9–24·0% from (+)-7:3′:4′-trihydroxyflavan-3:4-diol under the same conditions.

The tannin reddened rapidly in solution or in the solid form when exposed to sunlight. It was readily soluble in methanol and acetone, and moderately in ethanol and water.

Methoxyl derivative of (−)-leuco-fisetinidin tannin. Tannin (500 mg.) in methanol (100 ml.) was methylated with excess of ethereal diazomethane at −10° for 24 hr. The solution was reduced to small volume (2–3 ml.), under vacuum, treated with charcoal and filtered. The filtrate poured into water gave a white amorphous powder that did not crystallize from ethanol or methanol. The product (400 mg.) dried at 110° for 2 hr. had $[\alpha]_D^{21} - 30{\cdot}2 \pm 0{\cdot}2°$ in acetone (*c*, 0·9) (Found: C, 66·9; H, 6·2; OMe, 28·5. $C_{15}H_{11}O_3(OMe)_3$ requires C, 65·1; H, 6·1; OMe, 28·0%).

Heating the methylated tannin in 3N-HCl–propan-2-ol (1:4) under pressure gave a low yield of a red anthocyanidin (blue with ammonia), R_F 0·68 in 3N HCl–90% formic acid (2:1) and 0·95 in the (1:1) mixture. An anthocyanidin with similar R_F values is formed from (+)-7:3′:4′-trimethoxyflavan-3:4-diol.

A separate sample was methylated under identical conditions (Found: C, 66·5; H, 6·3; OMe, 29·4%), and the molecular weight, determined in acetone (three estimations) by the Ray (1952) ebulliometric method as modified by Evelyn (1954) and Evelyn, Cooper & van Berge (1954), was 1087±23; $[C_{15}H_{11}O_3(OMe)_3]_3$ requires 996.

The infrared-absorption curve of the methylated tannin was similar to that of (+)-7:3′:4′-trimethoxyflavan-3:4-diol over the range 2·5–15 μ and reaffirmed the absence of carbonyl groups in the tannin.

Acetyl derivative of (−)-*leuco-fisetinidin tannin.* Tannin (400 mg.) was acetylated with acetic anhydride (2·0 ml.) and pyridine (1·6 ml.) for 6 hr. at room temperature and poured into water. The white amorphous product (450 mg.) was redissolved in acetic acid and filtered into water. The flocculent product dried at 50° for 8 hr. over P_2O_5 had $[\alpha]_D^{21}$ +8·3±0·2° in tetrachloroethane (*c*, 1·2) (Found: C, 60·5; H, 4·6; $CO{\cdot}CH_3$ by acid hydrolysis, 37·8, 37·0%; mol.wt., 1539±16. $[C_{15}H_{10}O_2(CO{\cdot}CH_3)_4]_3$ requires C, 60·2; H, 4·8; $CO{\cdot}CH_3$, 37·6%; mol.wt. 1374). The infrared-absorption curve (KBr) over the range 2·5–15 μ was similar to that of penta-acetyl-(+)-mollisacacidin.

Acetylation of methylated (−)-*leuco-fisetinidin tannin.* Tannin (100 mg.) previously methylated with diazomethane (28·5% of methoxyl groups) was treated with pyridine (0·4 ml.) and acetic anhydride (0·5 ml.) at room temperature for 6 hr. The product (90 mg.) was isolated by the method used for acetylated (−)-leuco-fisetinidin tannin, dried at 110° for 2 hr. and had $[\alpha]_D^{21}$ +12·2±0·8 in acetone (*c*, 0·8) (Found: C, 65·0, 65·4; H, 6·0, 6·2; $CO{\cdot}CH_3$, 6·1; OMe, 20·6. $C_{29}H_{22}O_7$ requires C, 64·2; H, 5·9; $CO{\cdot}CH_3$, 11·5; OMe, 24·9%). Acetylation under the same conditions for only 2 hr. gave a product of lower acetyl-group but correspondingly higher methoxyl-group content (Found: $CO{\cdot}CH_3$, 2·3; OMe, 27·7%). The infrared-absorption curve (6·1% of acetyl groups) was almost identical with that of the methylated tannin except for additional peaks at 1725, 1360 and 1230 cm.$^{-1}$ associated with carbonyl and acetate groups.

Hydrogenation of (−)-*leuco-fisetinidin tannin.* The tannin was unchanged when treated with hydrogen in the presence of palladium catalyst under conditions (Weinges, 1958) used by Roux & Paulus (1961*a*) to convert (+)-7:3′:4′-trihydroxyflavan-3:4-diol into (−)-7:3′:4′-trihydroxyflavan-3-ol [(−)-fisetinidol].

Distribution of (−)-*leuco-fisetinidin tannin in wattle heartwood*

Examination of the heartwoods of a 50- to 60-year old freshly felled specimen of *A. mearnsii* by radial sampling and two-way paper chromatography (solvent combination 1) showed that the tannin was formed in the peripheral heartwood and was present throughout the heartwood. The tannin disappears slowly during aging of the wood after felling, and is absent from wood kept for 2–3 years, probably owing to atmospheric oxidation.

Synthesis of (±)-7:3′:4′-*trihydroxyflavan*-4-*ol*

(±)-Butin (500 mg.), synthesized from resacetophenone and protocatechuic aldehyde via the cyclization of butein (Saiyad, Nadkarni & Wheeler, 1937), was hydrogenated with platinum oxide (300 mg.) in methanol for 6 hr.; 105·5 ml. of H_2 was absorbed. A paper chromatogram of the methanolic solution showed the presence of a single substance of higher R_F (0·30) than (±)-butin (0·22) in 2% (v/v) acetic acid, and of lower R_F (0·83) than (±)-butin (0·92) in water-saturated butan-2-ol.

The methanol was removed under vacuum in a rotary evaporator on a water bath at 40–50°, and the viscous residue dissolved in 2–3 ml. of cold water and filtered. Crystals (200 mg.) deposited slowly from the filtrate when kept at 0° for 2–3 days. After drying over P_2O_5 under vacuum these melted at 94–96° with reddening, and the red melt decomposed sharply at 118–120°. Recrystallization from water does not alter either the m.p. or decomposition point (Found: C, 59·2; H, 6·2; loss at 80°, 8·5. $C_{15}H_{14}O_5$, $1{\cdot}5H_2O$ requires C, 59·8; H, 5·7; H_2O, 9·0%). After drying at 80° over P_2O_5 under vacuum (Found: C, 65·2; H, 5·5. $C_{15}H_{14}O_5$ requires C, 65·7; H, 5·2%).

The colours given with spray reagents were: bisdiazotized benzidine, yellow; ferric alum, olive green; silver nitrate, grey–black; toluene-*p*-sulphonic acid, deep blue fading to pale pink. The flavan-4-ol reddened on heating, on exposure to sunlight in the solid state or in solution, and on the addition of warm dilute HCl.

An intense blue–purple was produced after brief heating of the flavan-4-ol with 3N-HCl–propan-2-ol (1:4) under pressure at 100°. The colour persisted if the mixture was cooled immediately, but faded rapidly on continued heating.

(±)-7:3′:4′-Trimethoxyflavan-4-ol. The hydrogenation product of (±)-butin (500 mg.) in methanol (100 ml.) was methylated overnight with excess of diazomethane in ether at −10°, and the solvents were subsequently removed in a rotary evaporator on a warm (40–50°) water bath. The product crystallized readily from ethanol (4 ml.) at room temperature in clusters of white needles (275 mg.), m.p. 135–137°. Recrystallizing from ethanol increased the m.p. to 139–140° (Found: C, 68·0; H, 6·6; OMe, 29·8. $C_{18}H_{20}O_5$ requires C, 68·3; H, 6·4; OMe, 29·4%). The infrared-absorption curve of (±)-7:3′:4′-trimethoxyflavan-4-ol showed strong OH absorption at 3475 cm.$^{-1}$, but no absorption at 1665 and 1345 cm.$^{-1}$ frequencies characteristic of the C=O group in trimethyl-(±)-butin. A spot of 7:3′:4′-trimethoxyflavan-4-ol placed on paper from methanol solution and sprayed with ethanolic toluene-*p*-sulphonic acid reagent turned deep blue–violet at 60° and red on more prolonged heating at higher temperatures, or on aging at room temperature.

(±)-4-Acetoxy-7:3′:4′-trimethoxyflavan. (±)-7:3′:4′-Trimethoxyflavan-4-ol (100 mg.) was treated with 0·4 ml. of pyridine and 0·4 ml. of acetic anhydride at room temperature for 5 min. and poured into water. The oily product solidified rapidly and crystallized slowly from ethanol or ethanol–water in fine needles, m.p. 115–118° with sintering at 85–90° (Found: C, 67·0; H, 6·2; $CO{\cdot}CH_3$, 11·2; OMe, 26·9. $C_{19}H_{22}O_6$ requires 67·0; H, 6·2; $CO{\cdot}CH_3$, 12·0; OMe, 26·0%).

Comparison of colour and condensation reactions

Spraying chromatograms of the flavan-4-ol, flavan-3:4-diol and tannin with toluene-*p*-sulphonic acid with subsequent heating at 90° caused all the compounds to assume a final pink. For the flavan-4-ol and flavan-3:4-diol, the pink was preceded by a blue, which was very intense with the flavan-4-ol and less pronounced with the flavan-3:4-diol. The flavan-3-ols [(+)- and (−)-fisetinidols] gave no reaction. Toluene-*p*-sulphonic acid reagent gave

Table 1. *Condensation and colour reactions of flavans related to 7:3′:4′-trihydroxyflavan-4-ol*

Compound	Period before first appearance of condensation* product	Colour of condensation product	Colour of soln.
(±)-7:3′:4′-Trihydroxyflavan-4-ol	Instantaneous	Red–brown	Deep mauve→red
(+)-7:3′:4′-Trihydroxyflavan-3:4-diol	30 sec.	Light pink	Deep pink
(−)-7:3′:4′-Trihydroxyflavan-3-ol	10 min.	Buff	Colourless
(+)-7:3′:4′-Trihydroxyflavan-4-one-3-ol	No product	†	Pale yellow‡
(−)-7:3′:4′-Trihydroxyflavan-4-one	No product	†	Pale orange–yellow§
(+)-Catechin	5 min.	Buff‖	Colourless‖
(+)-7:3′:4′:5′-Tetrahydroxyflavan-3:4-diol	5–10 sec.	Light pink	Pink

* Where condensation was not immediate the elevated temperature (60°) was maintained to promote the reaction.
† White crystals consisting of unchanged material separate on cooling.
‡ Colour due to conversion into fisetin.
§ Colour due to conversion into butein.
‖ Previously shown by Bate-Smith & Swain (1953).

similar colours with the trimethyl ethers of the flavan-4-ol (blue turning to pink) and of flavan-3:4-diol (pink). A positive test is obtained even in the absence of a *p*-hydroxyl group in the 7-position, and therefore the reagent appears specific for the secondary benzyl alcohol (4-hydroxyl) grouping in flavonoids, and indicates that (−)-leuco-fisetinidin tannin contains this grouping.

Solid (±)-7:3′:4′-trihydroxyflavan-4-ol turns red rapidly when exposed to sunlight. Such reddening is also shown by (+)- and (−)-7:3′:4′-trihydroxyflavan-3:4-diol and by the leuco-fisetinidin tannin, but not by (+)- or (−)-7:3′:4′-trihydroxyflavan-3-ol, (+)-fustin or (−)-butin.

The analogues of (±)-7:3′:4′-trihydroxyflavan-4-ol when dissolved (3 mg.) in 1 ml. of boiling water and treated immediately with 1 ml. of cold 10N-HCl gave the condensation and colour reactions shown in Table 1. Similar reactions of (+)-catechin and (+)-7:3′:4′:5′-tetrahydroxyflavan-3:4-diol are included for comparison.

DISCUSSION

The heartwood of *A. mearnsii* contains a number of polymeric tannins of the condensed type. These are already present at the heartwood periphery where the concentration of the monomeric (+)-7:3′:4′-trihydroxyflavan-3:4-diol is at a maximum (Roux & Paulus, 1960), but those of very low R_F and high molecular weight increase towards the heartwood centre, corresponding with a decline of the flavan-3:4-diol (Roux & Paulus, 1960; Roux & Evelyn, 1960). This suggests a progressive transformation of the flavan-3:4-diol into condensed tannins of various degrees of condensation. The concentration of other flavonoid compounds present, for example (+)-fustin and (−)-fisetinidol, do not undergo such marked changes (cf. Roux & Paulus, 1960, 1961*a*). The presence of leuco-fisetinidin tannins in the peripheral heartwood is also found in *Schinopsis* spp. (Roux, 1958*b*; Roux & Evelyn, 1958*b*; Roux & Paulus, 1961*b*), indicating that some of the associated flavan-3:4-diols undergo relatively rapid transformation into tannins [cf. Freudenberg (1960)].

The leuco-fisetinidin tannin isolated in the present study exists in a colourless reduced form in the wood, and shows absorption maxima at 205 and 280 mμ typical of the flavans and their constituent phenols. This differentiates it immediately from the dark oxidation polymers ($\lambda_{max.}$ 280 and 420 mμ) containing repeating quinonoid groups, which were obtained by Hathway & Seakins (1957) and shown to be present in the commercial extracts prepared from *Acacia catechu* heartwood and *Uncaria gambir* leaves.

The tannin isolated shows similarity to (+)-7:3′:4′-trihydroxyflavan-3:4-diol in colour reactions, degradation products and ultraviolet- and infrared-absorption curves, but also marked differences. For example, the tannin appears to be trimeric and the yield of fisetinidin chloride obtainable from it approximates to a third of the yield of anthocyanidin from the flavan-3:4-diol. Calculated to a C_{15} basis, the tannin reacts with diazomethane to the equivalent of three methoxyl groups, corresponding to the methylation of all the phenolic hydroxyl groups in 7:3′:4′-trihydroxyflavan-3:4-diol. Direct acetylation affords an acetyl derivative containing the exact equivalent of four acetyl groups per C_{15} unit, and presumably represents the substitution of the equivalent of three phenolic and one aliphatic hydroxyl groupings. The tannin differs in this respect from the leucocyanidin isolated by Forsyth & Roberts (1960) from cacoa beans, where acetylation and methylation procedures both resulted in the substitution of an equal number of hydroxyl groups. Acetylation of the methylated derivative of the tannin causes demethylation, probably by direct substitution of methoxyl by acetyl groups, but without increase in the total number of hydroxyl groups substituted. This suggests that after methylation the substitution of the total equivalent of four hydroxyl groups, as obtained by direct acetylation, may be hindered owing to steric hindrance of the remaining hydroxyl grouping.

On the assumption that the tannin is a polymer of 7:3′:4′-trihydroxyflavan-3:4-diol, it is apparent that the equivalent of at least one aliphatic hydroxyl group per C_{15} unit is involved in direct condensation, or has undergone conversion into a tertiary alcohol as a result of condensation, or alternatively has become sterically hindered following condensation. The high negative rotation ($[\alpha]_D - 33°$) of the tannin in relation to the positive rotation ($[\alpha]_D + 12°$ to $+33°$) of the (+)-7:3′:4′-trihydroxyflavan-3:4-diol (cf. Clark-Lewis & Roux, 1959) is not understood at present, but the stereochemistry of the tannin holds future interest in view of the known interrelation between (−)-fisetinidol, (+)-fustin, (−)-butin and (+)-7:3′:4′-trihydroxyflavan-3:4-diol (Roux & Paulus, 1960, 1961*a*, *c*).

The reactivity of the 4-hydroxyl group in the flavan-3:4-diols has been predicted (Roux, 1959; Freudenberg, 1960) from a knowledge of lignin and lignin model substances, and is confirmed by a study of the properties of polymerization of (±)-7:3′:4′-trihydroxyflavan-4-ol in direct comparison with those of its analogues (Table 1). The ease of polymerization of flavan-4-ols and flavan-3:4-diols does not necessarily implicate only the 4-hydroxy substituent, since flavan-3-ols are shown to condense with relative ease and closely related flavans apparently condense at different rates [compare (+)-7:3′:4′-trihydroxyflavan-3:4-diol with (+)-7:3′:4′:5′-tetrahydroxyflavan-3:4-diol, and (−)-fisetinidol with (+)-catechin]. Nevertheless, the abnormally high reactivity of the flavan-4-ol and flavan-3:4-diol compared with the flavan-3-ol and other analogues (Table 1) emphasizes the high reactivitiy of the *sec.*-(*p*-hydroxybenzyl) alcohol group. This, together with the ease of hydrolysis of the tannin to the anthocyanidin with mineral acid, suggests that the 4-position is at least partly implicated in linking of flavonoid units in the tannin.

The flavan-4-ol also reddens when exposed to direct or indirect sunlight. Such reddening is typical of flavan-3:4-diols and of condensed tannins present in wattle wood and bark (*A. mearnsii* and related spp.), blackwood (*Acacia melonoxylon*), quebracho wood and bark (*Schinopsis* spp.), mangrove bark (*Rhizophora* spp.) and *Eucalyptus* spp. but does not occur with catechins (flavan-3-ols) or with condensed tannins from *A. catechu* heartwood and *U. gambir* leaves. This behaviour suggests that the hydroxyl group in the 4-position is responsible for redness (as distinct from brownness developed on oxidation) inherent in condensed tannins of the leuco-anthocyanidin type.

The synthesis of (±)-7:3′:4′-trihydroxyflavan-4-ol from the corresponding flavan-4-one affords an easy method of obtaining hydroxylated flavan-4-ols. The reduction will, however, be inhibited, where present, by a 5-hydroxyl group, owing to hydrogen bondage between this and the 4-carbonyl group (cf. Freudenberg & Weinges, 1958). The reduction also has stereochemical aspects and must be considered in conjunction with catalytic reduction of natural (±)-fustin to (±)-7:3′:4′-trihydroxyflavan-3:4-diol under identical conditions (Roux & Freudenberg, 1958). The 2-phenyl and 3-hydroxyl groups of (±)-fustin (2:3-*trans*-configuration) may be assigned the 2(*eq*), 3(*eq*) conformation suggested for natural flavanonols by Mahesh & Seshardi (1955). The *O*-trimethyl derivative of the reduction product of (±)-fustin easily forms an *iso*propylidene derivative and the parent flavan-3:4-diol was therefore assigned a 2:3-*trans*–3:4-*cis*-configuration (Clark-Lewis & Roux, 1959). This suggests a 2(*eq*), 3(*eq*), 4(*ax*) conformation for the (±)-7:3′:4′-trihydroxyflavan-3:4-diol (cf. King & Clark-Lewis, 1955; Chandorkar & Kulkarni, 1957), although a 4-hydroxyl group in the equatorial position would be expected from slow reduction under neutral conditions (cf. Barton, 1953) unless it assumed that the 4-carbonyl group is strongly hindered by the α-hydroxyl group in natural fustin. A study of molecular models shows that this hindrance may at most be described as partial.

(±)-Butin [2(*eq*)-phenyl conformation] may similarly afford a (±)-7:3′:4′-trihydroxyflavan-4-ol of 2(*eq*)-phenyl–4(*ax*)-hydroxyl conformation and 2:4-*trans*-configuration from catalytic reduction with platinum oxide in neutral solution, although the alternative of a 4(*eq*)-hydroxyl group cannot be excluded. The former would be related stereochemically to the natural (+)- and (−)-7:3′:4′-trihydroxyflavan-3:4-diols, their artificial racemate being identical with the reduction product of (±)-fustin (Clark-Lewis & Roux, 1959). This is believed to be the first synthesis of a flavan-4-ol containing a number of free phenolic hydroxyl groups, although Freudenberg (1956) cites a 7:4-dihydroxyflavan of which details have not been described.

SUMMARY

1. An optically active condensed tannin has been isolated from the heartwood of black wattle. The tannin resembles the accompanying (+)-7:3′:4′-trihydroxyflavan-3:4-diol in most chemical and physical properties, but is trimeric on a C_{15} basis.

2. Evidence available suggests that the tannin is a polymer of 7:3′:4′-trihydoxyflavan-3:4-diol condensed possibly through the heterocyclic ring system.

3. (±)-7:3′:4′-Trihydroxyflavan-4-ol has been synthesized from (±)-butin by catalytic hydrogenation. The stereochemical significance of the reduction is discussed.

4. The properties of the flavan-4-ol in comparison with those of the flavan-3-ol suggest that the hydroxyl group in position 4 might be involved in the biochemical condensation of flavan-3:4-diols, and also in the redness developed in these compounds and in associated or related condensed tannins.

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Diphosphopyridine Nucleotide Pyrophosphorylase in the Nuclei Isolated from Poisoned and Regenerating Rat Liver

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The stimulus for this work was a paper by Morton (1958) in which it was suggested that, since diphosphopyridine nucleotide is required for more enzymic reactions than any other known coenzyme, alteration of its rate of synthesis could modify cell division and differentiation and perhaps lead to malignant growth. An important enzyme in its synthesis is diphosphopyridine nucleotide pyrophosphorylase, which is located exclusively in the nuclei (Hogeboom & Schneider, 1952). Low activities of this enzyme were obtained in the nuclei from spontaneous mouse-mammary-gland carcinoma, hepatomas after feeding aminoazobenzene, Ehrlich ascites cells, post-operative human tumours and foetal mouse liver (Morton, 1958; Branster & Morton, 1956). These findings were correlated with

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the low diphosphopyridine nucleotide content of these tissues, and it was suggested that in rapidly growing cells synthesis of new cell material occurs against a limiting supply of diphosphopyridine nucleotide from the nucleus. When the diphosphopyridine nucleotide falls to a critically low concentration, the cells divide, thus providing more nuclear enzyme in relation to the cytoplasm.

It therefore seemed interesting to examine the diphosphopyridine nucleotide pyrophosphorylase of the nuclei from livers from animals after various poisons causing cell necrosis and also from livers regenerating after partial hepatectomy. Before this work was attempted an examination was made of isolation procedures for nuclei. Some modifications were made and some properties of nuclei obtained were compared with those obtained in other laboratories.

EXPERIMENTAL

Abbreviations. The exchange of ^{32}P (added as inorganic phosphate) with the phosphorus atoms of ATP, ^{32}P–ATP exchange reaction; deoxyribonucleic acid phosphorus, DNA P.

Materials

Animals. Female rats (150–200 g.) were used throughout. Sprague–Dawley rats were used for the experiments where DPN synthesis was measured and albino rats of the Porton strain for all other experiments. The animals were allowed free access to food at all times.

Special chemicals. The following chemicals were obtained from the sources indicated: disodium salt of ATP, nicotinamide mononucleotide, alcohol dehydrogenase (Sigma Chemical Co., St Louis, Mo., U.S.A.); nicotinamide and ethionine (Roche Products). Beryllium sulphate, dimethylnitrosamine, CCl_4 and ethionine were used as hepatotoxic agents. Partial hepatectomy was performed by the procedure of Higgins & Anderson (1931).

Methods

Isolation of nuclei. The nuclei were isolated by a modification of the basic technique of Hogeboom, Schneider & Striebich (1952). Schneider & Petermann (1950) showed that nuclei which were isolated in 0·25 M-sucrose agglutinate, but that it is possible to prevent this by adding small amounts of bivalent ions. They used 1·8 mM-$CaCl_2$ in 0·25 M-sucrose and calcium has been used in most procedures (Hogeboom *et al.* 1952; Allfrey, Mirsky & Osawa, 1957; Creasey & Stocken, 1959). It was, however, shown in the original work of Schneider & Petermann that magnesium could replace calcium. We have preferred to use magnesium as a more usual intracellular ion. The concentrations of magnesium used were empirically determined and were the maximum that could be used without causing gross agglutination of the mitochondria, thus preventing major contamination of the nuclei with mitochondria.

After decapitation of the rats, the livers were perfused *in situ* through the inferior vena cava with about 30 ml. of ice-cold 0·9% NaCl solution followed by 60–80 ml. of ice-cold 0·25 M-sucrose containing 0·4 mM-$MgCl_2$. The lobes were manipulated during perfusion in order to remove blood. In some of the poisoned rats (after dimethylnitrosamine and to a less extent after ethionine) it was impossible to obtain completely blood-free livers. The livers were removed, roughly dried, weighed and homogenized in 30 ml. of 0·25 M-sucrose containing 3 mM-$MgCl_2$, in a Potter–Elvehjem-type homogenizer with a smooth glass tube and a Perspex pestle (Aldridge, Emery & Street, 1960), with a gap of 0·01 in. (difference in diameter, 0·02 in.) and a speed of 1900 rev./min. Homogenization was complete after 15 up-and-down movements. The resulting homogenate was diluted to a volume (ml.) equal to 10 times the weight of the liver (g.) with 0·25 M-sucrose containing 3 mM-$MgCl_2$ and then filtered through one layer of flannelette (W.F. 12, J. H. Bounds Ltd., Manchester) to remove connective tissue and unbroken cells. A portion (10 ml.) of the filtered homogenate was layered over 20 ml. of 0·34 M-sucrose containing 0·4 mM-$MgCl_2$ and centrifuged for 10 min. at 700*g* at 0° in a swing-out head. The supernatant was pipetted off the sedimented material, which was resuspended in 5 ml. of 0·25 M-sucrose containing 0·4 mM-$MgCl_2$. Sucrose (10 ml. of 0·34 M) containing 0·4 mM-$MgCl_2$ was layered under this suspension and was centrifuged as before. The supernatant was removed and the procedure repeated. The final sediment was resuspended in 1 ml. of 0·25 M-sucrose containing 0·4 mM-$MgCl_2$/10 ml. of 10% (w/v) homogenate. All manipulations were carried out as near to 0° as possible.

Succinic dehydrogenase. This was determined by a modification (Aldridge & Johnson, 1959) of the manometric method of Quastel & Wheatley (1938). Dilution of the fraction with water before assay was essential to obtain maximum activity.

Adenosine triphosphatase. This was determined by the procedure described by Aldridge & Stoner (1960).

Protein. This was determined by the modified (Aldridge, 1957) biuret method of Robinson & Hogden (1940) and the results are expressed as albumin.

Deoxyribonucleic acid. This was determined by the diphenylamine method of Dische (1930), modified by Burton (1956).

Diphosphopyridine nucleotide pyrophosphorylase. This was determined by a modification (Branster & Morton, 1956) of the original method of Kornberg (1950). The medium contained, in 0·8 ml., 0·3 ml. of 0·25 M-glycylglycine buffer brought to pH 7·4 with KOH, 5 μmoles of ATP, 3 μmoles of nicotinamide mononucleotide, 300 μmoles of nicotinamide, 15 μmoles of $MgCl_2$ and 0·2 ml. of a suitable suspension of nuclei. The mixture was shaken for 20 min. at 38° in air and the reaction was stopped by the addition of 0·8 ml. of N-$HClO_4$. Blanks were made either at zero time by adding the nuclei after the $HClO_4$ or by omitting the nicotinamide mononucleotide from the reaction mixture. No difference was found between the two blanks and low values for DPN content were always obtained. After chilling in ice, the tubes were centrifuged at 0°. The sediment was resuspended in N-$HClO_4$ and recentrifuged. The supernatants were combined, brought to approx. pH 8 by adding NaOH, with phenol red as an internal indicator, and diluted to 2·5 ml.; 1 ml. was used for the determination of DPN by using alcohol dehydrogenase as described by Kornberg (1950). DPN formation in 20 min. was linearly related to the amount of nuclei used and with a fixed amount of nuclei was linear with time.

RESULTS

Properties of the nuclear fraction

Contamination of the nuclear fraction. There are two major difficulties in obtaining pure suspensions of nuclei. One is contamination with whole liver cells and erythrocytes and the other is contamination by mitochondria. In our experience the conditions of homogenization to produce complete disruption of all liver cells also causes extensive damage to the nuclei. The minimum clearance and maximum speed of rotation of the pestle has therefore been chosen to produce minimal damage to the nuclei as judged by their microscopical appearance. Erythrocytes have been removed by perfusion of the liver and the major amount of intact whole cells has been filtered off with flannelette. As discussed later this is not entirely satisfactory for some purposes, but preliminary experiments with a fine nylon bolting cloth have been promising. The bivalent ions used to preserve the nuclear membrane can cause agglutination of mitochondria. Contamination of nuclei with mitochondria (other than those in whole cells) is caused by such agglutination and subsequent sedimentation with the nuclei. The maximum concentration of magnesium chloride that will cause minimal sedimentation of the mitochondria with the nuclei has been used in the various solutions. The contamination by whole liver cells and mitochondria has been determined by measurements of succinic dehydrogenase. The final procedure adopted gives suspensions of nuclei containing 1–1·7 % of the succinic dehydrogenase of the original homogenate (Table 1). Determinations of the ^{32}P–ATP exchange reaction in the suspension of nuclei gave an activity that could be accounted for by a contamination with less than 2 % of the activity of the mitochondria in the original homogenate. In common with the ^{32}P–ATP exchange in mitochondria (Boyer, Luchsinger & Falcone, 1956; Löw, Siekevitz, Ernster & Lindberg, 1958), the ^{32}P–ATP exchange mediated by the suspension of nuclei was inhibited by 2:4-dinitrophenol. The simplest explanation of these findings is that there is a slight contamination by mitochondria or whole cells, equivalent to 1–2 % of the original homogenate, and that the nuclei do not contain a system of oxidative phosphorylation comparable with that in mitochondria.

Nuclear size. The preparations of nuclei were examined microscopically either as fresh suspensions by phase contrast or as smears stained by the Feulgen technique. Both methods showed most of the nuclei well shaped, round, with one or more

Table 1. *Succinic dehydrogenase, the [^{32}P]phosphate–adenosine triphosphate exchange reaction and adenosine triphosphatase in nuclear fractions from rat liver*

The adenosine triphosphatase measurements were determined on the same preparation as in Expt. 1 under succinic dehydrogenase.

Succinic dehydrogenase

Expt. no.	Homogenate	Nuclear fraction	Percentage in nuclear fraction
	(μl. of CO_2/min./g. wet wt. of liver)		
1	435	7·4	1·7
2	284	3·4	1·2

Adenosine triphosphatase

Additions	Homogenate	Nuclear fraction	Percentage in nuclear fraction
	(μg.atoms of P/hr./g. wet wt. of liver)		
Nil	573	47·5	8·3
	(μg.atoms of P/hr./mg. of protein)		
Nil	3·22	6·55	—
33 μM-2:4-Dinitrophenol	5·93	7·45	—

^{32}P–ATP *exchange reaction*

Additions	Mitochondrial fraction	Nuclear fraction	Percentage in nuclear fraction
	(counts/min./μg.atom of exchangeable P/g. wet wt. of liver)		
Nil	20 100*	353	1·7
33 μM-2:4-Dinitrophenol	—	65	—

* Taken from Aldridge & Threlfall (1961).

visible nucleoli. No cytoplasmic tags were adherent to the nuclei. The preparations were contaminated by a few unbroken cells, nuclear fragments and a few mitochondria and very occasionally red cells.

Experiments were carried out to determine the effect of varying the concentration of sucrose in the suspending medium upon nuclear diameter. The results are given in Fig. 1 and show that nuclear diameter varies inversely with the concentration of sucrose. The value for nuclear diameter in 0·25 M-sucrose is 8·1 μ which agrees with the values of 8·26 μ and 7·5–8·5 μ given for normal nuclei measured in fixed and stained material by Abercrombie & Harkness (1951) and Rather (1958) respectively. These results indicate that a concentration of sucrose of about 0·25 M is suitable for the isolation of nuclei, though the accuracy of this procedure is not such that it is possible to say that 0·25 M is better than 0·3 M.

Formation of 'nuclear gels'. Damage to mitochondria releases an enzyme that has the property of preventing the formation of gels when the nuclei are subsequently treated with alkali at pH 9–10 (Dounce, Witter, Monty, Pate & Cottone, 1955; Dounce & Monty, 1955). These workers consider that DNA is normally bound firmly to some protein component by non-ionic linkages and that it is only by exposure to damaged mitochondria that these linkages are broken. On this view the formation of gels by treatment with alkali may be considered a property of 'normal' nuclei. The preparation in this paper formed very quickly a transparent viscous gel when the pH was brought to 9–10.

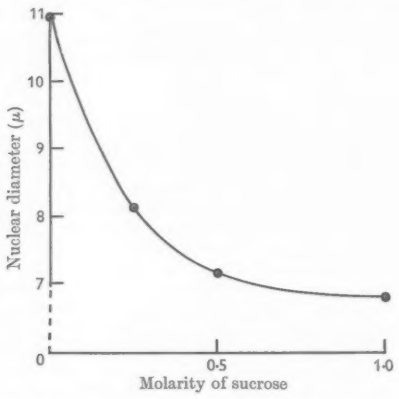

Fig. 1. Mean diameter of rat-liver nuclei suspended in sucrose of various molarities. The nuclei were suspended in the sucrose solutions for 1 hr. at 0° before measurement of their diameter with a phase-contrast microscope with a calibrated eyepiece. At each concentration of sucrose 60–80 measurements were made.

Adenosine triphosphatase in nuclear fractions. Although several workers have measured adenosine triphosphatase activity in nuclear fractions, there are difficulties in the interpretation of the results. By both the non-aqueous method of isolation (Behrens, 1939; Allfrey, Stern, Mirsky & Saetren, 1952) and that with aqueous solutions (Lang & Siebert, 1951; Philpot & Stanier, 1956; Chauveau, 1952) preparations from liver and kidney are obtained which contain adenosine triphosphatase activity (Stern, Allfrey, Mirsky & Saetren, 1952; Novikoff, Hecht, Podber & Ryan, 1952; Lang & Siebert, 1951; Fischer, Siebert & Adloff, 1959; Miller & Goldfeder, 1960). In most cases there is much doubt either about the degree of contamination by other constituents of the cytoplasm or about the effects of the solvents used in the non-aqueous method upon such enzyme(s). Nuclei prepared by the method described in this paper have appreciable adenosine triphosphatase activity (Table 1), the specific activity of the nuclei being higher than that of the homogenate. Succinic-dehydrogenase determinations indicate a contamination of the nuclear fraction by 1·7 % of the mitochondria of the homogenate. In contrast the nuclear fraction contains over 8 % of the adenosine triphosphatase. The addition of 2:4-dinitrophenol stimulated the activity of the homogenate by 85 %, but the nuclear fraction by only 8 %. The slight stimulation of the nuclear fraction is not more than 1·4 % of the stimulation obtained in the homogenate and is presumably due to the mitochondria. It is clear therefore that our nuclear fraction contains an adenosine triphosphatase not associated with mitochondrial contamination, and therefore rat-liver nuclei contain an adenosine triphosphatase.

Yield of nuclei. The efficiency of the recovery of nuclei from the homogenate by centrifuging is best judged by determinations of DNA. The DNA recovered in three experiments was 66, 55 and 70 % of that present in the original unfiltered homogenate. We have been unable satisfactorily to determine DNA in the filtered homogenate. As far as we can deduce from our experiments, this appears to be due to the elution from the flannelette of some substance that interferes in the method of assay of DNA but which does not persist to the final suspension of nuclei. Further experiments indicate that nylon bolting cloth is suitable as a filtration material without the above disadvantages. With this nylon bolting cloth the recovery of DNA from the filtered homogenate is almost quantitative (90–100 %). It is certain therefore that a good recovery of nuclei is obtained from the homogenate filtered through flannelette. The quotient,

Table 2. *Diphosphopyridine nucleotide pyrophosphorylase in nuclei isolated from rats after various treatments*

Table 2. *Diphosphopyridine nucleotide pyrophosphorylase in nuclei isolated from rats after various treatments*

All substances were injected as solutions in 0·9% NaCl solution, except CCl_4 which was as a 10% (v/v) solution in arachis oil. i.v., Intravenous; i.p., intraperitoneal; s.c., subcutaneous; oral, stomach tube.

Treatment and reference	Dose (mg./kg. body wt.)	Time killed	Protein in nuclear fraction (mg./g. wet wt. of perfused liver)	*P*	DPN pyrophosphorylase in nuclear fraction (μmole of DPN/mg. of protein/hr.)	*P*
Control	—	—	7·4±0·99 (5)	—	0·228±0·026 (5)	—
Beryllium (*a*)	0·6 (i.v.)	24 hr.	8·0±0·85 (4)	0·4	0·170±0·037 (4)	0·03
Dimethylnitrosamine (*b*)	50 (i.p.)	24 hr.	7·3±1·0 (4)	0·9	0·206±0·024 (4)	0·2
CCl_4 (*c*)	160 (oral)	24 hr.	7·1±1·1 (4)	0·7	0·189±0·041 (4)	0·13
Ethionine (*d*)	187 (i.p. twice daily)	24 hr.	7·6±2·2 (4)	0·8	0·208±0·052 (4)	0·5
Thioacetamide (*e*)	30 (s.c. daily)	12–13 days	5·2±0·75 (4)	0·01	0·224±0·024 (4)	0·8
	30 (s.c. daily)	18 days	6·4 (2)*		0·233 (2)	
Nicotinamide (*f*)	500 (i.p.)	3 hr.	6·3 (2)		0·226 (2)	
	500 (i.p.)	12 hr.	7·8±0·4 (3)	0·6	0·204±0·022 (3)	0·9
Partial hepatectomy (*g*)	—	24 hr.	4·0±0·5 (4)	<0·001	0·185±0·016 (4)	0·02
	—	48 hr.	5·4±1·3 (4)	0·04	0·147±0·013 (4)	<0·001
	—	72 hr.	5·1±0·76 (4)	0·006	0·106±0·019 (4)	<0·001

* This value is the mean of 4·6 and 8·3. The latter is undoubtedly too high owing to a loss of diluting fluid during isolation. This will not, however, influence the specific-activity measurements.

(*a*) Aldridge, Barnes & Denz (1949), Cheng (1956). (*b*) Barnes & Magee (1954). (*c*) Cameron & Karunaratne (1936). (*d*) Koch-Weser, Farber & Popper (1951). (*e*) Gupta (1956). (*f*) Bonsignore & Ricci (1958), Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach (1956). (*g*) Higgins & Anderson (1931).

protein/DNA P (mg. of protein as albumin/mg. of DNA P), is 49 for the nuclear fraction.

Diphosphopyridine nucleotide pyrophosphorylase in nuclei from livers of rats after various treatments. In Table 2 are the results of assays of DPN phosphorylase in the nuclear fractions isolated from animals after various treatments.

DISCUSSION

For studies of this kind, nuclei should be obtained from liver in the state in which they are present in the liver cells. To achieve this perfection may be an impossibility but, unlike our knowledge of mitochondria (Aldridge, 1957), we have little or no knowledge of what criteria to adopt in order to judge the state of our preparations. The procedure in this paper has been to test our preparations with properties that have been suggested by various authors. Qualitatively this preparation seems to fulfil these criteria. Many treatments of nuclei cause them to lose protein thus lowering the quotient, protein/DNA P (Allfrey *et al.* 1952). Chauveau, Moulé & Rouiller (1956) obtained a value of 40 for nuclei isolated by high-density sucrose solutions, whereas values of 61 and 78 were obtained for calf- and horse-liver nuclei respectively with the non-aqueous method (Allfrey *et al.* 1952). The value for our preparation of approx. 49 falls between these values. However, the high values obtained by the latter authors were calculated by using a value for protein obtained by subtracting the DNA and RNA from the total dry weight.

DPN-pyrophosphorylase activity in the isolated nuclei has been determined by the formation of DPN from nicotinamide mononucleotide and ATP. However, recent work has indicated that DPN is not formed in this way *in vivo*. Priess & Handler (1958*a*) have shown that, in human erythrocytes and in rat liver *in vivo*, after the administration of nicotinic acid the intermediates found are nicotinic acid mononucleotide and deamido-DPN. These intermediates have also been found after the administration of nicotinamide (Langan, Kaplan & Shuster, 1959; Threlfall, 1959). At the enzymic level we have to consider whether the reactions whereby nicotinamide mononucleotide is converted into DPN and nicotinic acid mononucleotide is converted into deamido-DPN are catalysed by the same enzyme. Nicotinic acid mononucleotide-pyrophosphorylase and DPN-pyrophosphorylase activities are closely associated at all stages of Kornberg's (1950) method of purification (Priess & Handler, 1958*b*), and the distribution of the two activities between the various subcellular fractions from rat liver is also very similar (Priess & Handler, 1958*b*). The available evidence therefore indicates that the enzymic activity measured in this work is the

same enzyme which converts nicotinic acid mononucleotide into deamido-DPN *in vivo*. Therefore, contrary to the scheme put forward by Morton (1958), the enzyme measured is not now the terminal enzyme in the synthesis of DPN *in vivo*. At the final enzymic step deamido-DPN is converted into DPN.

Obvious damage, including nuclear changes, is present in rat liver 24 hr. after the administration of dimethylnitrosamine (Barnes & Magee, 1954), carbon tetrachloride (Cameron & Karunaratne, 1936) and beryllium (Aldridge, Barnes & Denz, 1949; Cheng, 1956). After the single doses of ethionine, fatty livers are found without any necrotic changes (Koch-Weser, Farber & Popper, 1951). After 14–21 daily doses of thioacetamide the nuclei in many of the liver cells are almost double their normal size with the single nucleolus almost as big as a normal-size nucleus (Gupta, 1956). It is particularly striking that in this work we have found little change in the DPN-pyrophosphorylase activity of the nuclei isolated from livers of animals so treated. Only after injection of beryllium is the change statistically significant (P 0·03); but these are marginal changes, particularly since Cheng (1956) has reported that changes in the nuclei were visible 5 hr. after dosing. Even with grossly abnormal nuclei (after injection of thioacetamide) there is no change in the specific activity of the nuclei. These results indicate that this enzyme is not suitable as an indicator of nuclear damage or change, for it is apparent that such morphological changes are not accompanied by changes in enzyme activity. This situation may be compared with mitochondria and succinic dehydrogenase. This enzyme is very stable and so firmly attached to the mitochondrial structure that no early changes are to be expected with mitochondrial damage. An example of the contrary situation with change in enzyme activity in nuclei with presumably no morphological changes has been reported. The DPN-pyrophosphorylase activity of livers of mice (Waravdekar, Powers & Leiter, 1956*a*, *b*) and rats (Ono & Tomaru, 1959) bearing tumours at other sites is much lower than that of controls. This contrasts with the normal DPN concentration in the livers from tumour-bearing rats (Glock & McLean, 1957).

After administration of dimethylnitrosamine the DPN pyrophosphorylase of liver nuclei is normal. C. J. Threlfall (unpublished work) has shown that after dimethylnitrosamine the DPN content of the liver falls rapidly to 50 % of the control value. However, after injection of nicotinamide, the DPN content of the liver rises by the same absolute amount in both treated and control animals. This indicates that DPN synthesis is unaffected in animals after dimethylnitrosamine and our results are consistent with this view.

After partial hepatectomy the DPN-pyrophosphorylase activity of the nuclei isolated from the regenerating liver decreased continuously for 3 days after operation. During this period the liver is increasing in size and reaches its original weight in 7 days (Abercrombie & Harkness, 1951). A similar low DPN pyrophosphorylase has been found in foetal mouse liver and in liver during the rapid growth after birth (Branster & Morton, 1956). There are few values for the DPN contents of either foetal or regenerating liver with which to compare these results. In the rat low values in embryonic liver (30 % of the concentration in adult liver) and a continuously rising content up to normal adult values in 7 days have been reported (Jedeikin, Thomas & Weinhouse, 1956). The concentrations of DPN in regenerating liver examined 3 days after partial hepatectomy were 20 % lower than the controls. De Burgh (1957) has reported that, although there was no consistent change in the DPN concentration in regenerating mouse liver, after the injection of nicotinamide the increase in DPN in the liver was 65 and 39 % of the controls 1 and 2 days respectively after the removal of the liver. Neglecting the difference in species, these latter results show the same trend as ours.

After partial hepatectomy the remaining liver increases to double its weight in roughly 3 days (Abercrombie & Harkness, 1951; Hammersten, Åqvist, Anderson & Eliasson, 1956; Nygaard & Rusch, 1955). With the results of Abercrombie & Harkness (1951), the total enzyme activity remaining in the liver after operation and for each succeeding day may be calculated. The results are necessarily approximate and depend on the assumption that the rate of regeneration is the same as that obtained by Abercrombie & Harkness (1951) and also that the percentage recovery of nuclei is the same for each preparation. The total enzyme activity obtained by such a calculation is less on all 3 days after operation than that present in the liver remaining immediately after operation. This is a surprising result and indicates that no synthesis of enzyme (other than that necessitated by normal turnover) has occurred. In contrast, DNA in regenerating liver follows the protein and liver weight, both of which double in 3 days (Åqvist & Anderson, 1956; Hecht & Potter, 1956). The activity of many enzymes in regenerating rat liver has been measured (Harkness, 1961), but with few exceptions unfractionated liver homogenates were used. One exception, the cathepsin activity of isolated liver nuclei during regeneration, has been studied (Maver, Greco, Løvtrup & Dalton, 1952). In contrast with DPN pyrophosphorylase the specific activity of the nuclei was considerably higher than that of the controls, 2 and 3 days after operation.

SUMMARY

1. The procedure of Hogeboom *et al.* (1952) for the isolation of liver nuclei has been modified.

2. Some properties of the isolated nuclei have been examined. They do not catalyse a [^{32}P]phosphate–adenosine triphosphate exchange reaction but they do contain more adenosine triphosphatase activity than can be explained by contamination with mitochondria.

3. Little change was found in the specific activity of diphosphopyridine nucleotide pyrophosphorylase of nuclei isolated from livers of rats poisoned with the hepatotoxic agents beryllium, carbon tetrachloride, dimethylnitrosamine and ethionine.

4. After partial hepatectomy, the specific activity of diphosphopyridine nucleotide pyrophosphorylase of nuclei isolated from the regenerating liver falls continuously for 3 days to approximately half of its control value.

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The Estimation of Androst-16-en-3α-ol in Human Urine

PARTIAL SYNTHESIS OF ANDROSTENOL AND OF ITS β-GLUCOSIDURONIC ACID

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Androst-16-en-3α-ol (II) was originally isolated, together with the 3β-epimer, from pig's testes by Prelog & Ruzicka (1944); the structure of the stenols was elucidated by Prelog, Ruzicka & Wieland (1944), who prepared other Δ^{16}-C_{19} steroids and drew attention to their remarkable odours. Other Δ^{16}-compounds in the C_{19} and C_{18} series have been synthesized, but only the androst-16-en-3-ols have been detected in biological extracts; indeed the 3β-ol has so far been found only in pig's testes. The 3α-ol was obtained from the urine of normal men and women by Brooksbank & Haslewood (1949, 1950, 1952), after treatment of a glucosiduronate extract with β-glucuronidase, in amounts which suggested that it was excreted at a rate of about 1 mg./day: the method of isolation seemed to exclude the possibility of its being a chemical artifact. Androstenol (about 15 mg./day) has also been isolated from the urine of a female patient with an adrenal tumour (Mason & Schneider, 1950), of a patient with a luteoma of the ovary (Engel, Dorfman & Abarbanel, 1953) and of a patient with adrenal hyperplasia (Miller, Rosenkrantz & Dorfman, 1953).

Burstein & Dorfman (1960) have recently shown by isotope studies in a woman with a virilizing adrenal tumour that (II) can be formed from cholesterol via 3β-hydroxypregn-5-en-20-one. All this work suggests that (II) is metabolically related to the well-known androgens, as of course its chemical structure implies. However, in some ways (II) is of unusual interest. Its occurrence in normal urine in much more than trace quantities points to its being more than an insignificant, chancy, metabolic by-product. If the biochemical precursor of (II) is a C-17-hydroxy compound, as at first sight seems likely, its formation would involve a dehydration reaction, hitherto regarded as rare and very little studied in the steroid series in mammalian systems: if the precursor is not a C-17-hydroxy compound, its origin presents an even more intriguing biochemical problem. Finally, the powerful smell of (II), described by many as 'musk-like', and its formal structural resemblance to civetone might conceivably have far-reaching physiological implications.

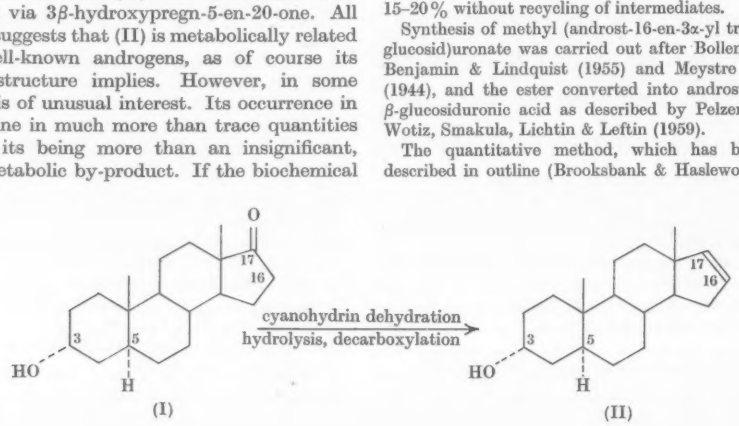
It therefore seemed worth while to begin an investigation of the metabolism of (II) by a study of its urinary excretion in health and disease. This paper describes, first, a partial synthesis of the free steroid and of its β-glucosiduronic acid, and, secondly, the details and trials of a method of estimation, including preliminary quantitative data on urinary excretion in normal men and women.

GENERAL METHODS

The partial synthesis of androst-16-en-3α-ol (II) from androsterone (I) was carried out as described in a preliminary communication (Brooksbank, Haslewood, Pollock & Hewett, 1958). In a small-scale pilot synthesis, the overall yield was about 34%; on a larger scale, it was 15–20% without recycling of intermediates.

Synthesis of methyl (androst-16-en-3α-yl tri-*O*-acetyl-β-glucosid)uronate was carried out after Bollenback, Long, Benjamin & Lindquist (1955) and Meystre & Miescher (1944), and the ester converted into androst-16-en-3α-yl β-glucosiduronic acid as described by Pelzer (1959) and Wotiz, Smakula, Lichtin & Leftin (1959).

The quantitative method, which has been already described in outline (Brooksbank & Haslewood, 1960), is



(I) (II)

designed to be simple enough for routine application, consistent with adequate specificity for androst-16-en-3α-ol. The opportunity has been taken to establish accuracy by using the β-glucosiduronic acid of androstenol for recovery experiments from urine. This refinement, which obviates uncertainties about the completeness of hydrolysis of conjugates, has not often been applied to analysis of urinary steroids conjugated with glucuronic acid.

EXPERIMENTAL

General. Elementary analysis were by Weiler and Strauss, Oxford. Melting points are uncorrected. The symbols E_{520} and E_{575} refer to extinctions measured with (green) and (yellow) light filters whose peak transmissions are stated to be at 530 and 575 mμ and transmission ranges 440–630 and 550–630 mμ. respectively. The filter transmitting maximally at 575 mμ was suitable for measurement of the colour given by androstenol in the conditions described below. For correction of E_{575} (see below) it was advantageous to use a green filter of wide transmission range.

Partial synthesis of androst-16-en-3α-ol

3α-Acetoxy-5α-androstan-17-one cyanohydrin. The cynaohydrin mixture was prepared from androsterone acetate as by Ruzicka, Plattner, Heusser & Pataki (1946). The amorphous white solid obtained was dried *in vacuo* over $CaCl_2$. No attempt was made to obtain a pure cyanohydrin.

3α-Acetoxy-17β-cyanoandrost-16-ene. The dehydration of the cyanohydrin with $POCl_3$ was carried out exactly as described by Ruzicka *et al.* (1946), but by using dry Carius tubes heated in a furnace. The dried cyanohydrin was processed in this way with batches of 1–2 g. each per Carius tube. After recrystallization of the *3α-acetoxy-17β-cyanoandrost-16-ene* from aqueous acetone, the final (constant) m.p. was 201–204·5° (Found: C, 77·8; H, 9·1; N, 4·1. $C_{22}H_{31}O_2N$ requires C, 77·4; H, 9·2; N, 4·1%). A typical yield from 5·15 g. of androsterone acetate was (after one recrystallization) 3·10 g. of the 17-cyano compound, i.e. 58·5%.

3α-Hydroxyaeti-16-enic acid. The hydrolysis of the 17-cyano compound was done in small metal bombs. For example, to 3α-acetoxy-17β-cyanoandrost-16-ene (326 mg.) in a bomb were added ethanol (8·8 ml.) and N-NaOH (4·4 ml.). The sealed bomb was heated at 180–185° for 5 hr. The pooled product from seven batches (total wt., 2·58 g.) was diluted with water to about 340 ml. and the mixture extracted with ether. (Evaporation of the washed and dried ether left a neutral material, which could be subjected to a second hydrolysis, with or without further treatment with HCN.) The alkaline aqueous phase was acidified with HCl, and, after an interval, the crude aetienic acid was collected by filtration. The filtrate (after addition of excess of NaCl) and precipitate were extracted with ethyl acetate, and the extract was washed with water, dried (Na_2SO_4) and evaporated to give the crude aetienic acid. Yield (after desiccation), 1·90 g. (79·6%).

3α-Hydroxyandrost-16-ene (see also Sondheimer, Mancera, Urquiza & Rosenkrantz, 1958). The above crude 3α-hydroxyaeti-16-enic acid (2·15 g.) was boiled for 4 hr. in quinoline (39 ml., freshly distilled from KOH) with the addition (in two portions) of activated copper chromite catalyst (389 mg. in all, as described by Adkins & Connor, 1931). After the addition of water (1660 ml.), the mixture was extracted repeatedly with ether. The ethereal extract was washed with aqueous HCl, NaOH and water, dried (Na_2SO_4) and evaporated. The yield of neutral material (crude androstenol) was 1·48 g. (79·9%). Unchanged aetienic acid was recovered from the alkaline phase. Pure 3α-hydroxyandrost-16-ene could be obtained in good yield by sublimation of the crude neutral material at 125–135°/0·3 mm. Hg. The crystalline sublimate was recrystallized from aqueous acetone to (constant) m.p. 144·5–146°, not depressed by authentic material. As many different batches of crude androstenol were worked up together, it was not possible to calculate yields of pure androstenol in the larger-scale experiments. But in a typical sublimation, 186 mg. of crystalline sublimate were obtained from 216 mg. of crude material (yield 86%); in another experiment, 136 mg. of crude material yielded 65 mg. (m.p. 140–145·5°) after sublimation and recrystallization (yield 48%). The overall yield of pure androstenol from androsterone was usually about 15–20%. As the amount of side products formed at any stage in the synthesis seemed to be small, this yield could probably be substantially improved by recycling material through the process: this was in fact done to some extent.

Preparation of androst-16-en-3α-yl β-glucosiduronic acid

Methyl (androst-16-en-3α-yl 2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate (cf. Meystre & Miescher, 1944). Androst-16-en-3α-ol (200 mg.) in anhydrous benzene (25 ml.) containing Ag_2CO_3 (750 mg.) was treated dropwise during 1·5 hr. with a solution of methyl α-bromotri-*O*-acetylglucuronate [750 mg.; m.p. 103–105°; $[\alpha]_D^{23} + 189 \pm 1°$ in $CHCl_3$ (*c*, 2·7); prepared by slight modification of the procedure of Bollenback *et al.* (1955)] in anhydrous benzene (40 ml.). Continuous distillation of benzene (in all, about 40 ml.) was carried out for a total time of 2 hr. After all the bromo compound had been added the mixture was refluxed for a further 30 min. The benzene solution was filtered and evaporated. The dried residue was treated with ethanol–water (70:30, v/v). Crystalline material was obtained, which after recrystallization (twice) from aqueous ethanol had m.p. 171–172° (Found: C, 65·2; H, 7·9. $C_{32}H_{46}O_{10}$ requires C, 65·1; H, 7·8%). Yield, 117 mg. of material of m.p. > 164° (27%).

Androst-16-en-3α-yl β-D-glucopyranosiduronic acid (cf. Pelzer, 1959; Wotiz *et al.* 1959). The above methyl (androstenyl tri-*O*-acetylglucosid)uronate (m.p. 166–171°; 87 mg.) in ethanol (1·5 ml.) was treated with 0·49 N-$Ba(OH)_2$ (5 ml.). The mixture was heated for 2–3 min. on a boiling-water bath and then kept overnight at 5°. The precipitated barium salt was filtered off and washed with ice-cold water and with acetone. It was dissolved in methanol (9 ml.) containing N-HCl (0·15 ml.), and the gently boiling solution was treated dropwise with 0·1 N-H_2SO_4 (1·5 ml.). After removal of the precipitated $BaSO_4$ by centrifuging, the supernatant was filtered, concentrated under a N_2 stream at 40° and cooled to 0–5°. The yield of white solid which separated was enhanced by addition of water; the solid was isolated by centrifuging. On slow recrystallization from a mixture of ethanol (1·5 ml.) with water (4·5 ml.) there was obtained *androst-16-en-3α-yl β-D-glucopyranosiduronic acid* of m.p. 169–174° (yield 37 mg.; 56%) [Found: androstenol,

60·7 (method described below); glucurone, 38·7 (method of Fishman & Green, 1955, slightly modified); C, 65·9, H, 8·7. $C_{25}H_{38}O_7$ requires androstenol, 60·8; glucurone, 39·2; C, 66·7; H, 8·4%].

Quantitative estimation of urinary androstenol

Apparatus. The columns for chromatography were of length, 200 mm. and diameter, 10 mm., with 0·5–0·7 mm. bore at the jet; they were fused to 150-ml. round-bottomed flasks at the top and fitted with flat-type stopcocks to facilitate adjustment of running rates. All glassware was of standard-joint and kept scrupulously clean; particular care was taken to avoid contamination by bench-wax and similar materials.

Reagents. These were as follows. Ether: anaesthetic ether, B.P., was redistilled from fresh bottles, or recovered by distillation from spent ether after washing with $FeSO_4$ and water. The ether was tested for peroxide with vanadic acid before use. Ethanol: for standard solutions, etc., was purified by shaking with *p*-phenylenediamine and redistilled. Methanol: for rinsing glassware, was redistilled. Benzene: was dried with Na and redistilled. Light petroleum: dried with Na and the fraction of b.p. 83–97° was used. Acetic acid: A.R., was treated with acetic anhydride and CrO_3 (Weissberger & Proskauer, 1935), and redistilled. Sulphuric acid: A.R., was N-free. Resorcylaldehyde: 2:4-dihydroxybenzaldehyde, was recrystallized from ether–light petroleum (b.p. 80–100°) and from water under N_2. Alumina: type H (Peter Spence and Co. Ltd., Widnes, Lancs.) was equilibrated in 1 kg. batches with 3–4% (v/w) of water by mechanical shaking for 6 hr. The partially deactivated alumina was stored in a calibrated dispenser to minimize entry of atmospheric water vapour during sampling. β-Gluronidase: was prepared from limpets after Dodgson & Spencer (1953) to powder *B* stage. To improve the yield, powder *A* (120 g.) was extracted twice with water (400 ml.), which was then treated with acetone (1200 ml.) Assay figures for the β-glucuronidase activity of powder *B* were obtained by the method of Fishman, Springer & Brunetti (1948), scaled down to conserve substrate, at final concentrations of powder of 10–20 μg./1·5 ml. of incubation mixture.

Method

Hydrolysis. Urine (24 hr. specimens) is collected in glass (not plastic) bottles and sent to the laboratory as soon as possible. The volume is noted, and, if from an adult and less than 1 l., made up to 1 l. with water. No preservative is used until the urine is received in the laboratory, when 1 ml. of a solution of $Hg(CN)_2$ (0·2 g./100 ml. in water)/100 ml. of urine is added. Duplicate samples taken for analysis are 1/20 of the 24 hr. vol. for urines of young adult men and 1/10 of the 24 hr. vol. for others. The samples are adjusted with 5N-HCl (a few drops) to approximately pH 4·5 and to each 100 ml. are added 4M-sodium acetate buffer, pH 4·5 (6·25 ml.) and 100 000–150 000 Fishman units of limpet β-glucuronidase [40–60 mg. of Dodgson & Spencer's (1953) powder *B*]. The samples are then incubated at 37–40° for 65–66 hr.

Extraction. The incubated urine is extracted with 1·0, 0·5 and 0·5 vol. of ether, and the combined ethereal extract washed with N-NaOH (0·1 vol.) and water (3 × 0·1 vol.). Care is taken to rinse precipitated material thoroughly in the incubation flask with ether, and to rinse alkali from the stopper of the separating funnel. A few drops of Bradosol (Ciba Ltd.; 5%, w/v) may be added, if necessary, to prevent emulsions. The washed ether extract is dried with Na_2SO_4 (6–7 g., freshly dried) for 1–2 hr. and filtered into a long-necked round-bottomed flask, containing three glass-beads; the Na_2SO_4 and filter paper are rinsed carefully with dry ether (2 × 25 ml.) without delay. The ether is evaporated rapidly at atmospheric pressure, not quite to dryness, on a boiling-water bath, with the flask set at an angle of about 45° and carrying a splash-head. The last drops of solvent are evaporated under reduced pressure in a vacuum desiccator (containing $CaCl_2$), in which the extract is stored at atmospheric pressure. The details of this evaporation procedure and the subsequent evaporation described below must be carefully followed, in view of the volatility of androstenol.

Chromatography. The column of alumina is prepared by filling the glass column with eluent mixture and running in alumina (5 g.) from the dispenser on to a small plug of fat-free cotton-wool set in the constriction of the column; the liquid is at the same time allowed to run out and the column is tapped to drive out air bubbles and to level the surface. A layer (5 mm. thick) of acid-washed sand is placed above the alumina.

Only one eluent mixture, light petroleum (b.p. 83–97°)–benzene (1:1, v/v), is used throughout. Androst-16-en-3α-ol (about 300 μg.) is dissolved in this mixture and the solution is used to determine the volume of eluent required for each batch of deactivated alumina, as follows. The androstenol in eluent mixture (4 + 2 + 2 ml.) is run into a column of alumina prepared as described above and then eluted. The eluate, running at 30–40 ml./hr., is collected automatically as 2-ml. fractions in tubes (0·5 in. diam.). Solvent is evaporated in an oven and the colour reaction applied to the residues. The apparent activity of the alumina, in terms of the elution volume for androstenol, is not quite constant during the first 4 weeks or so after the preparation of the alumina, and, apart from testing each new batch, checks should be made from time to time on the same batch especially when a new lot of eluent mixture is made up. However, with a 'safety margin' of 10–15 ml. of eluent on each side of the determined elution range of androstenol, and adherence to the procedure described, no losses of androstenol on the alumina columns need be expected.

The dry urinary extract, prepared as described under Extraction, above, is leached with 4, 2, and 2 ml. of eluent mixture, and each rinse carefully and separately applied and run into a fresh alumina column. Two fractions are then eluted as above, the second, containing the androstenol, being collected in a 150 ml. long-necked round-bottomed flask. [The volumes of eluent used for these two fractions will depend on the elution volume of androstenol; the volumes should be in the ranges 25–50 ml. for the first fraction (excluding the 8 ml. used for application of the extract) and 75–85 ml. for the second fraction.] The 'androstenol fraction' is evaporated, if necessary the following day, on a water bath at 80–90° under reduced pressure (water-pump). To prevent losses, the long-necked flask is fitted with a splash-head, and set at an angle of about 45° only just dipping into the hot water; three glass beads are added and the flask is tapped gently until boiling is quite steady. As soon as evaporation is complete, the flask is immediately removed from the bath and evacuated in a

vacuum desiccator at about 22° until the last traces of eluent are removed (about 5 min.). The flask is then kept in the desiccator at atmospheric pressure.

Colorimetry. The dry 'androstenol fraction' is dissolved in the 150 ml. flask in 3·0 ml. of purified acetic acid containing resorcylaldehyde (0·5 g./100 ml.), the neck of the flask being carefully rinsed down. To allow for complete and homogeneous solution, the mixture is kept for 10 min. in the stoppered flask with occasional mixing. Two 1·0 ml. samples are then pipetted into colour reaction tubes (10 cm. × 1 cm.) and 1·0 ml. of acetic acid–H_2SO_4 mixture (95:5, v/v) is added to each tube, with careful mixing. 'Standards' of pure androstenol are conveniently prepared by evaporating measured samples of a stock solution (500 μg./ml. in ethanol) in colour reaction tubes over $CaCl_2$ in a vacuum desiccator, and dissolving the residues in 1·0 ml. of acetic acid–resorcylaldehyde reagent. 'Blanks' consist of 1·0 ml. samples of acetic acid–resorcylaldehyde reagent in colour reaction tubes. 'Standards' and 'blanks' are each treated as above with 1·0 ml. of acetic acid–H_2SO_4 mixture. In view of the reproducibility and linearity of the 'standard' calibration curves, a pair of identical 'standards', containing 20–30 μg. of androstenol, will usually suffice for each series of estimations.

The tubes are placed without delay in a water bath at 100° for 8 min. The size of the tubes is not critical, and stoppering is unnecessary, but the bath should be such that the liquid in the tubes is fully immersed in the water and out of direct illumination, while the mouths of the tubes are protected from condensing steam. A convenient arrangement for 10 cm. × 1 cm. tubes consists of two perforated metal plates, 7 cm. apart in the water, the lower supporting the tubes, the upper resting on top of the bath and having holes 1·2 cm. in diameter through which the tubes pass. After heating, the tubes are cooled in ice–water and kept in the dark. After about 20 min., the coloured solutions are diluted with 2·0 ml. of acetic acid and measured spectrophotometrically at about 575 and 530 mμ. (With an EEL photoelectric colorimeter, suitable filters are Ilford Spectrum Yellow, no. 626 and EEL Green, no. OGR1.) The corrected extinctions at about 575 mμ [E_{575} (corr.)] may be found from the observed extinctions less 'blank' [E_{575} (obs.)] by using the expression: E_{575} (corr.) = $[2E_{575}$ (obs.) $- E_{530}]/0{\cdot}95$, where E_{530} is the extinction, less 'blank', at about 530 mμ (for explanation, see Results). If any other colour filter combination is used a different expression, empirically determined, is of course applied. As slight spectral changes occur on standing, it is advisable to measure the colours after very approximately the same interval. The 'blank' should have an extinction of not more than 0·01 in 1 cm. tubes. Smaller colorimeter tubes can be used, as it is not necessary to dilute the colour mixture after heating. When colours giving E_{575} (corr.) > 0·4 are obtained, the androstenol content should be calculated by reference to a calibration curve: E_{575} (corr.) *versus* μg. of androstenol (see Fig. 2 and Results).

RESULTS

Evaluation of the method of analysis

Colour reaction. The colorimetry is a quantitative adaptation of one of the reactions described by Miescher (1946) for 17-hydroxy and Δ^{16}-C_{19} steroids. The final concentrations chosen for the resorcylaldehyde and for the H_2SO_4 (2·5 vol. %) represent the best compromise found between maximum specific colour and minimum 'blank' colour. The purple colour obtained shows maximal absorption at 580–585 mμ and an inflexion at 520–540 mμ (Fig. 1): Fig. 1 also gives the absorption spectra of the 'blank', of a chromatographic eluate from a urinary extract of average apparent androstenol content, and of one from an extract giving only a trace of the androstenol purple colour.

Heating for 8 min. was found to be optimum. Provided the coloured reaction mixture was then kept in the dark, the extinction at about 580 mμ remained constant for at least 3 hr. whereas the extinction at about 530 mμ fell by about 3–5 % in this time. Fading of the colour occurred rather more rapidly after dilution with acetic acid. In daylight, the colours faded much more rapidly before than after dilution, whereas exposure to bright sunlight (through glass) caused a complete change in under 20 min. to a pinkish colour with absorption maximum at 555 mμ.

The relationship between intensity of colour and concentration of androstenol is shown in Fig. 2. The molecular extinction coefficient, ϵ_{585}, is 11 850. As Fig. 2 shows, the extinctions at about 575 mμ are linear over a wider range of concentration than are those at about 530 mμ and the ratio E_{530}/E_{575} rises at extinctions greater than 0·4.

The data of Miescher (1946) on the specificity of the colour reaction have been confirmed and

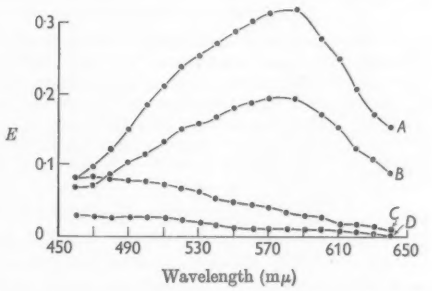


Fig. 1. Absorption spectra of colours obtained in the resorcylaldehyde–H_2SO_4–acetic acid reaction. Curve *A*, 29 μg. of pure androst-16-en-3α-ol; curve *B*, a typical urinary androstenol fraction; curve *C*, a urinary androstenol fraction containing almost entirely non-specific chromogens; curve *D*, the reagent 'blank'. Reaction mixture contained: resorcylaldehyde (0·5 %, w/v) in acetic acid (1 ml.) and H_2SO_4 (5 %, v/v) in acetic acid (1 ml.); acetic acid (2 ml.) was added after heating; extinctions were measured in 1 cm. cuvettes on a Unicam SP. 600 spectrophotometer against water; figures for curve *D* are subtracted from those for curves *A–C*.

extended. The following steroids occurring in urine extracts gave no visible colour at the concentration of 10–20 μg. in 2 ml. of reaction mixture: androsterone, dehydro*epi*androsterone (as acetate), 6β-hydroxy-3:5-*cyclo*androstan-17-one, androsta-3:5-dien-17-one, androst-2-(or-3-)-en-17-one, 3β-chloroandrost-5-en-17-one, progesterone, 3β-hydroxypregn-5-en-20-one (as acetate) and tetrahydrocortisone. A larger amount (50 μg.) of free dehydro*epi*androsterone still gave no colour. About 100 μg. of androstane-3:17-dione and pregnane-3:20-dione gave a barely perceptible yellow, but 100 μg. of cholesterol (purified) gave a pink colour (ϵ_{540}, 978); 25 μg. of androstane-3β:17α-diol and of aetiocholane-3α:17α-diol respectively gave colours of intensities of 1·33 and 1·12 relative to that given by the same weight of androst-16-en-3α-ol; androstane-3β:17β-diol and aetiocholane-3α:17β-diol gave no colour at this concentration. It seems likely that no appreciable interference can be expected in the colour reaction by known urinary steroids of polarity similar to that of androstenol, with the possible exception of cholesterol.

The specificity of the method might be improved if the correction formula of Allen (1950) for non-specific chromogens were applicable. This possibility was carefully studied, but was rejected after consideration of the spectral characteristics of colours given by androstenol and interfering substances.

The fraction eluted from alumina columns as described above, and which should contain the androstenol of urine extracts, gave a colour which had not precisely the spectral characteristics of that generated from pure androstenol (Fig. 1): it was pinker and absorbed more at lower wavelengths, whereas the ratio (E_{530}/E_{575}) of the extinctions (as defined under Experimental and corrected for the 'blank') was greater than that for pure androstenol. Under conditions given above, the ratio E_{530}/E_{575} for pure androstenol was 1·05; and the highest ratio observed for a urinary fraction was 2·0. If the latter figure is taken as the limiting value for an extract containing representative interfering chromogens and no androstenol, and if the assumption is made that the colour due to the interfering chromogens obeys Beer's Law, then E_{575} (less 'blank') can be corrected by the formula (cf. Gibson & Evans, 1937):

$$E_{575}\ (\text{corr.}) = \frac{K_I E_{575}\ (\text{obs.}) - E_{530}\ (\text{obs.})}{K_I - K_A}$$

where $K_I = E_{530}/E_{575}$ (= 2·0, in our case, for interfering chromogen) and $K_A = E_{530}/E_{575}$ (= 1·05, for androstenol).

Hence:

$$E_{575}\ (\text{corr.}) = [2E_{575}\ (\text{obs.}) - E_{530}\ (\text{obs.})]/0{\cdot}95.$$

Table 1 shows the extent of this correction with typical urines. Obviously, the ideal of absolute

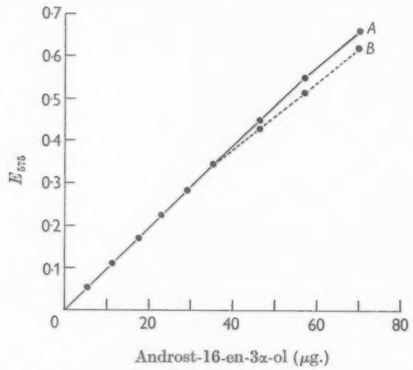


Fig. 2. Relation between μg. of androst-16-en-3α-ol and extinction (E_{575}, see text) without (*A*) and with (*B*) empirical correction as described in the text. Reaction mixture contained: resorcylaldehyde (0·5%, w/v) in acetic acid (1 ml.) and H_2SO_4 (5%, v/v) in acetic acid (1 ml.); acetic acid (2 ml.) was added after heating; extinctions were measured in 1 cm. diameter tubes in an EEL colorimeter.

Table 1. *Application of colour correction formula to urinary androstenol fractions*

Colorimetric extinctions with resorcylaldehyde–H_2SO_4–acetic acid reaction on urinary androstenol fractions with and without empirical correction for non-specific colour. For details, see text. Extinctions are less 'blank' as defined in text.

Type of extract	E_{575} (obs.)	E_{530}	E_{530}/E_{575}	Apparent androstenol (μg./24 hr.)*	E_{575} (corr.)	Corrected androstenol (μg./24 hr.)†
Low androstenol titre, much pink chromogen	0·058	0·085	1·47	186	0·033	99
	0·022	0·038	1·73	112	0·006	31
	0·062	0·114	1·84	109	0·011	37
Medium titre, moderate pink chromogen	0·157	0·180	1·15	546	0·141	495
	0·146	0·178	1·22	483	0·108	360
	0·078	0·090	1·16	489	0·070	438
High titre	0·163	0·179	1·10	1120	0·155	1040
	0·257	0·286	1·11	1760	0·240	1640
	0·423	0·457	1·08	1330	0·410	1340

* Calculated from E_{575} (obs.). † Calculated from E_{575} (corr.).

Table 2. *Recoveries of androstenol in later stages of the method described in the text*

	Amount added (μg.)	Amount recovered (%)	No. of tests
After chromatography on alumina, in pure solution	29·3	101, 104	2
	58·6	101, 101	2
	65·2	99, 99	2 (no control)
Added before evaporation of eluent mixture	29·0	100–103	9
Added to urine extract fraction (equivalent to 0·0375 of a 24 hr. specimen and containing the colour equivalent of 4·2 μg. of androstenol after chromatography and evaporation of eluent)	62·8	100–103	4

Table 3. *Effect of various amounts of β-glucuronidase and time of incubation on the yield of androstenol from pooled male urine*

Figures are for 100 ml. of urine.

Wt. of limpet powder *B* added (mg.)	β-Glucuronidase added (Fishman units)	Duration of incubation (hr.)	Androstenol found (duplicate results) (μg.)
37·5	100 000	66	77·9, 81·0
75	200 000	66	84·5, 83·6
75	200 000	44	79·4, 81·0
150	400 000	44	82·4, 83·1
150	400 000	22	69·3, 67·6

specificity is not achieved by this manoeuvre, but the corrected values are likely to be substantially nearer the true values than are those calculated from E_{575} alone. The occurrence of contaminants in urinary androstenol fractions seems to be capricious, depending upon the simultaneous presence of material extracted from the urine and impurities in reagents. We have found that the contaminating colour is usually not more than the equivalent of 50 μg. of androstenol/24 hr. (uncorrected E_{575}); it has rarely amounted to the equivalent of about 100 μg. No manoeuvre has yet been devised to eliminate this non-specific chromogenic material.

Chromatography of androstenol. Unfractionated neutral lipid extracts from urine gave a purple colour with the resorcylaldehyde–H_2SO_4–acetic acid reagent far more intense than could be accounted for by androstenol alone. Purification by some form of chromatography was therefore necessary. Androstenol could be separated readily from cholesterol and other very non-polar steroids in the paper-chromatographic system heptane–phenylethane-1:2-diol of Neher & Wettstein (1952), and separations could be achieved with reversed-phase partition chromatography on hydrophobic Celite. However, simple alumina-column chromatography was found effective and much easier to apply quantitatively. The procedure described under Method above was rigorously tested for possible variables and disturbing factors. It separated androstenol from most chromogenic impurities in the extract with a convenient elution volume.

The accuracy of the analyses in the chromatographic stages of the method is demonstrated by the results in Table 2.

Conditions for enzymic hydrolysis of androstenyl β-glucosiduronic acid. The availability of synthetic androstenyl β-glucosiduronic acid made possible the establishment of optimum conditions for the enzymic hydrolysis of the conjugate in urine. It was assumed that limpet glucuronidase will hydrolyse androstenyl β-glucosiduronic acid in urine buffered with acetate at pH 4·5, which is approximately optimum for hydrolysis of 17-oxo steroid β-glucosiduronic acids (Stitch & Halkerston, 1956). Tests of the best conditions were confined to those of enzyme concentration and durations of incubation. With β-glucuronidase made from limpets and assayed with phenolphthalein β-glucosiduronic acid, data as regards amount of enzyme and duration of incubation were determined and are listed in Table 3. It is apparent that a shorter time of incubation cannot be offset by adding more enzyme. An initial enzyme concentration of 1000 units/ml. of urine is probably adequate, as was confirmed in recovery experiments with added androstenyl β-glucosiduronic acid (Table 4), but the slightly larger amount of 1340 units/ml. of urine was chosen, for security.

Precision of the whole method. The conditions finally chosen, as well as the accuracy of the whole method, were checked in a series of recovery experiments (Table 4). For some of these, androstenyl β-glucosiduronic acid was added to urines from subjects who apparently excreted very small amounts of androstenol, in others a pool prepared by mixing eight assorted normal male and female specimens was used. In this way, reasonable checks were made against the possibility of any of these specimens, and particularly those from subjects with low androstenol excretion, containing abnormally large amounts of β-glucuronidase inhibitors.

The occasional occurrence of marked β-glucuronidase inhibition, as in any method employing

Table 4. *Recovery of androst-16-en-3α-ol, with the whole method as described in the text, after addition of androstenyl β-glucosiduronic acid to urine*

Androstenol is expressed as μg. of free androstenol/100 ml. of urine.

No. of determinations	Initial content of androstenol	Androstenol added	Recovery (%)		Enzyme units added/ml. of urine incubated
			Mean	S.D. (±)	
		(a) On low-content urine			
2	12·0	61·7	82·5	2·3	1600
2	10·2	61·7	88·5	1·5	1500
2	5·4	30·9	92	0	1340
2	5·4	61·7	86	2·0	1340
3	8·4	15·5	94	0·8	1340
3	8·4	30·9	99	4·1	1340
4	5·9	61·7	89	3·0	1340
3	11·7	15·5	95	8·6	1340
3	11·7	30·9	90	2·6	1340
2	47·4*	71·0	90	2·0	1340
2	53·7†	71·0	88·5	1·5	1340
2	10·2	61·7	89	3·0	1000
		(b) On higher-content (pooled) urine			
2	60·3	61·7	86·5	1·5	1500
3	55·5	30·9	96	0·7	1340
3	55·5	61·7	87	1·4	1340
2	60·3	61·7	85	2·0	1000

* Rotten urine sterilized by boiling before incubation. † Rotten urine not boiled before incubation.

Table 5. *Preliminary figures for urinary androstenol in normal human subjects*

Males		Females	
Age (yr.)	Androstenol (μg./24 hr.)	Age (yr.)	Androstenol (μg./24 hr.)
11	30	4	53*, <10
12	267	6	<10
13	162	9	<10
15	78	15	549
18	495, 501, 1330	16	1100
19	126, 105, 540, 1090, 1430, 1620, 1340	18	1010*, 431*, 450, 468
		19	698*, 509*, 72, 552
20	2040, 486, 1120, 921, 1960, 1080, 1160, 1280, 1910	20	500*, 423, 696, 642
		21	390
21	2040, 906, 846, 1610, 2630, 1870, 591	22	138
		23	819
22	1730, 547, 1480, 2330	25	411
23	1290	28	225, 417, 249
24	420	31	183
25	(947, 1044, 624)†, 544	37	66
27	1110	38	183
31	1130*	39	141*
34	(96, 19)†	69	258
35	1450	72	62
40	1450*	—	—
41	1390	—	—
45	805	—	—
53	228	—	—
71	540, 625	—	—
80	60	—	—

* Values from E_{575}, uncorrected by formula given in text.

† Values obtained on urines collected on different occasions, from the same person. All other values refer to different individuals.

this enzyme in urine, cannot of course be guaranteed, but the recovery figures show that incomplete hydrolysis of β-glucosiduronic acid conjugates, described, for example, by Gallagher *et al.* (1954), is unlikely to be serious with androstenyl β-glucosiduronic acid. For the 40 tests (Table 4), the overall mean percentage recovery and its standard deviation were 90·4 ± 5·3.

Androstenol excretion in normal subjects, preliminary results. In Table 5 are listed values obtained for the urinary output of material appearing in the same alumina chromatogram fraction as androst-16-en-3α-ol and giving the characteristic purple colour reaction. Some figures which were not corrected for non-specific colour are included, as the absolute reduction of the values incurred by the correction is small (Table 1).

DISCUSSION

Quantitative method for urinary androstenol. The recovery data show that the degree of precision is quite adequate for the purpose of obtaining information on the physiological significance of this steroid; the method is also simple to carry out. Drawbacks are the apparent volatility of the steroid and the chance development of interfering chromogens in the colour reaction.

The material which is estimated as androst-16-en-3α-ol and referred to as 'androstenol' is not of course proven to be the 3α:5α-epimer. However, other work has shown that androst-16-en-3β-ol, aetiochol-16-en-3α-ol and androsta-5:16-dien-3β-ol are not eluted from alumina in the 'androstenol fraction' of this method.

Contamination of urinary androstenol fractions by non-specific chromogens does not lead to serious inaccuracy except in urine extracts of very low androstenol content, and in any case its effect is greatly reduced by application of the colour correction formula as described above. Recoveries at the concentration of 15 μg. of androstenol/100 ml. of urine, where the actual amount of androstenol being estimated in the colorimeter tubes was about 8 μg., demonstrate that amounts of androstenol corresponding to 100 μg./24 hr. of urine can be distinguished from zero satisfactorily. Nevertheless, it is obviously advisable to repeat determinations on low-titre urines with a larger sample, to improve the precision of the analysis. In order to minimize contamination, particular attention must be paid to avoiding 'non-polar' organic material such as vaseline, silicone grease, bench wax etc.: it is also necessary to avoid the use of plastic bottles for urine collection, especially if organic solvents are added as preservatives.

As regards preservation of urine, we have found that a falling-off in the androstenol titre of a sample occurs in 1–2 weeks at about 22°, whereas at 5° a slight decrease may be apparent after about 6 weeks unless a preservative has been added. Urine samples need not, and should not, be boiled, for this also incurs some loss of androstenol.

The possible occurrence of androstenyl sulphate in urine was tested by incubation at 40° of an acidified ethyl acetate extract of sulphate conjugates (Burstein & Lieberman, 1958) from a mixture of male and female urines of varying androstenol glucuronide content. Added androstenol was recovered after this procedure, but no endogenous sulphate-conjugated androstenol was detected.

Normal values for urinary androstenol. Our data are insufficient to enable any firm conclusions to be made as to the physiological significance of this steroid. The very great variation, amounting to some 100-fold, between male subjects of the same age group is most striking. It is also clear that the actual amounts of androstenol excreted in the urine are not insignificant, being of the same order as that of the 5β-17-oxo steroids with oxygen functions at C-11 and only a little less than that of dehydro*epi*androsterone. In spite of this large variation between individuals, the mean figure so far found for women is under one-half of that for men, and only 15% of the male subjects so far investigated excreted less androstenol than the mean for women of reproductive age.

SUMMARY

1. The partial synthesis is described of androst-16-en-3α-ol from androsterone, through the dehydration of androsterone acetate cyanohydrin, saponification, and decarboxylation of the derived aeti-16-enic acid.

2. The synthesis of methyl (androst-16-en-3α-yl tri-*O*-acetyl-β-D-glucopyranosid)uronate and the preparation from it of androst-16-en-3α-yl β-D-glucosiduronic acid are described.

3. A method for the estimation of glucuronide-conjugated androst-16-en-3α-ol in human urine is described in detail. The procedure includes hydrolysis of the conjugate with β-glucuronidase, separation of the androstenol by chromatography on alumina, and quantitative application of a colour reaction employing resorcylaldehyde and sulphuric acid in acetic acid.

4. The specificity and limitations of the colour reaction have been studied.

5. The precision of the whole method has been evaluated. Recoveries of androstenol, added to urine as the pure β-glucosiduronic acid, exceeded 82% in the range 15–71 μg. of androstenol/100 ml. of urine and averaged 90·4 (S.D. ± 5·3)%.

6. Preliminary figures for androstenol excretion in urine are given. In the age group 18–45 years they ranged from 19 to 2630 μg./24 hr. in men and from 66 to 1010 μg./24 hr. in women.

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The Reaction of Mono- and Di-functional Alkylating Agents with Nucleic Acids

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In attempts to relate the chemical reactions of the alkylating agents with their biological effects two general principles have emerged. First, a general positive correlation has been established between chemical reactivity, as measured by rate of hydrolysis, and cytotoxicity (Haddow, Kon & Ross, 1948; cf. review by Ross, 1953). Secondly, the difunctional agents, i.e. those possessing two alkylating groups in the molecule, generally exert a markedly more powerful cytotoxic action than the corresponding monofunctional agents (Loveless & Ross, 1950). This has been ascribed to their ability to cross-link fibrous macromolecules, in particular those involved in duplication of the chromosomes (Goldacre, Loveless & Ross, 1949), reaction of this type between di-(2-chloroethyl) sulphide (mustard gas) and deoxyribonucleic acid having been proposed by Elmore, Gulland, Jordan & Taylor (1948). The importance of deoxyribonucleic acid as a site of biological alkylation was also suggested by observation of the mutagenic activity of mustard gas (Auerbach & Robson,

1946) and subsequently of a wide variety of alkylating agents (Fahmy & Fahmy, 1956). The difunctional agents are not markedly more effective as mutagens in comparison with the monofunctional, although differences in the types of mutation produced by these classes of agent have been found (Fahmy & Fahmy, 1961). If the principal biological effects of the alkylating agents are to be ascribed to their reaction with any one cellular constituent, the nature of this reaction must be such as to account for the relationship between chemical structure and biological activity that have been outlined above. The mode of combination of mustard gas with nucleic acids has been established and the reaction shown to occur *in vivo* (Brookes & Lawley, 1960). In order to ascertain whether the criteria for a reaction of biological significance are satisfied, a comparison of alkylating agents of different reactivities, and of mono- and di-functional agents of closely similar reactivities, seemed desirable. Investigations were therefore undertaken of the reactions with nucleic acids of ethyl iodide, diethyl sulphate, methyl methanesulphonate, ethyl methanesulphonate, 1:4-dimethanesulphonoxybutane (myleran), HN 2 [di-(2-chloroethyl)methylamine], nor-HN 2 [di-(2-chloroethyl)-amine], *NN*-di-(2-chloroethyl)aniline and 2-hydroxyethyl 2-chloroethyl sulphide. The binding of 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide and of ^{3}H-labelled myleran to cellular constituents in the mouse was also determined. A detailed comparison has been made of the reaction of nucleic acids with 2-hydroxyethyl 2-chloroethyl sulphide and with mustard gas, a preliminary account of which has been given (Brookes & Lawley, 1961*a*).

EXPERIMENTAL

Materials. ^{35}S-Labelled mustard gas (200 μc/mg.) and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (200 μc/mg.) and labelled starting materials for other radioactive preparations were obtained from The Radiochemical Centre, Amersham, Bucks.

^{3}H-Labelled myleran was prepared from but-2-ene-1:4-diol by catalytic reduction with tritium and had a specific activity of 3·1 c/g. ^{14}C-Labelled myleran (4·0 μc/mg.) was prepared by conversion of [2:3-^{14}C]succinic acid into 1:4-dihydroxy[2:3-$^{14}C_2$]butane (Mann & Nystrom, 1951) and subsequent reaction with methanesulphonyl chloride. Di-(2-chloro[$^{14}C_2$]ethyl)amine hydrochloride (3·6 μc/mg.) was prepared by reaction of [$^{14}C_2$]ethylene oxide (Cox & Warne, 1951) with ethanolamine and subsequent chlorination of the labelled diol with thionyl chloride. [1-^{14}C]Ethyl methanesulphonate (9·9 μc/mg.) was prepared by reaction of [1-^{14}C]ethyl iodide with silver methanesulphonate. *NN*-Di-(2-chloroethyl)[^{14}C]aniline (0·63 μc/mg.) was supplied by Dr J. J. Roberts and Dr G. P. Warwick.

Paper chromatography, radioautography and counting techniques. The methods were as described by Brookes & Lawley (1960).

Reaction of alkylating agents with nucleic acids in vitro. RNA or DNA was dissolved in aqueous buffer solution, containing sufficient potassium acetate or sodium phosphate to maintain neutrality during the reaction with the alkylating agents, to give solutions of concentration 0·1–0·01 M-nucleic acid P. The alkylating agent was added to the solution, which was maintained at 37°, and after appropriate times samples were taken and nucleic acid was precipitated with 2 vol. of ethanol containing 2% of sodium acetate. When radioactive alkylating agents were used the concentration was in the range 0·001–0·2 mole/mole of nucleic acid P and in other cases up to 1 mole/mole of nucleic acid P. Details of the concentrations used for individual alkylating agents are given in Table 1. The extent of alkylation was determined for labelled alkylating agents by assaying the radioactivity of the precipitated nucleic acids, and for unlabelled reagents by isolation and estimation of the products after acid hydrolysis of the alkylated nucleic acids. When labelled reagents were used the rate of alkylation was determined from the specific radioactivity of the nucleic acid precipitated at suitable time intervals during the course of the reaction (Table 1). With RNA the extent of alkylation increased to a maximum and then remained constant, whereas with DNA the specific radioactivity increased initially at a rate equal to that for RNA, but subsequently decreased, as for ^{35}S-labelled mustard gas (Brookes & Lawley, 1960).

Identification of the products of alkylation of nucleic acids. When ^{14}C- or ^{35}S-labelled alkylating agents were used a sample of alkylated DNA, sufficient to contain at least 5 μmc (up to 5 mg.), was hydrolysed with acid, and a radioautograph of a two-dimensional paper chromatogram prepared. The R_F values of the radioactive spots were compared with those of the expected 7-alkylguanines (Brookes & Lawley, 1961*b*).

In other cases the acid hydrolysate from alkylated nucleic acid was chromatographed on Dowex 50 (H^+ form), and the pyrimidine nucleotides and purine bases were eluted with HCl. The amounts of the unchanged purine bases and of the 7-alkylguanine products were estimated by summation of the extinctions at 260 mμ, and the products were identified by evaporation of the appropriate fractions followed by neutralization of the resulting hydrochlorides and crystallization of the free bases from water.

In a typical example, yeast RNA (240 mg.; 0·5 m-mole) was treated with ethyl methanesulphonate (62 mg.; 1 mole/mole of RNA P) in 0·4 N-potassium acetate buffer (5 ml.; initial pH 7·2) at 37° for 48 hr. RNA was precipitated with 2 vol. of ethanol containing 2% of potassium acetate and hydrolysed with N-HCl (5 ml.) for 1 hr. at 100°, and the resulting solution cooled and chromatographed on a column (17 cm. × 2 cm.) of Dowex 50 (H^+ form; equilibrated with N-HCl), with N-HCl as solvent. Fractions (50 ml.) were collected automatically and their ultraviolet absorption was measured at 260 and 280 mμ). Fractions 1–8 contained uridylic and cytidylic acids, fractions 16–24 contained guanine (0·134 m-mole), fractions 41–52 contained a product (0·01 m-mole) with ultraviolet-absorption spectra similar to those of 7-methylguanine, and fractions 64–100 contained adenine (0·1 m-mole). The fractions containing the 7-alkylguanine were evaporated, and the resulting hydrochloride was dissolved in the minimum of water and neutralized, when a crystalline base was deposited. This was shown by comparison of R_F values in

three solvents and ultraviolet-absorption spectra at pH 1, 7 and 12 to be identical with an authentic specimen of 7-ethylguanine (Brookes & Lawley, 1961*b*).

The results of experiments with labelled and unlabelled alkylating agents together with RNA (from yeast or rat liver) or DNA (from calf thymus) are shown in Table 1.

Hydrolysis of alkylated deoxyribonucleic acid in neutral aqueous solution. After the alkylation of DNA by di-(2-chloro-[^{14}C]ethyl)amine, [^{14}C]ethyl methanesulphonate, ^{14}C-labelled myleran, ^{35}S-labelled mustard gas or 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide, as described above, portions of the reaction mixture were taken after 120 hr. and the DNA was precipitated. The supernatant was evaporated and a two-dimensional paper chromatogram of the residue obtained. Radioautography of this chromatogram showed the presence of the 7-alkylguanines, which had been established as the products of the initial stages of the reaction.

A more detailed study of the liberation of the 7-alkylguanine products was made with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide. DNA (10 mg.) was dissolved in 0·01 N-phosphate buffer, pH 7·1 (2 ml.), at 37° and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (32 μg.) in ether (10 μl.) added. Samples (0·05 ml.) were withdrawn after 30 min. and subsequently at various intervals up to 12 days. The samples were applied to strips of Whatman no. 1 paper and chromatographed with ethanol–conc. NH_3 soln.–water (80:2:18, by vol.) as solvent. After drying, the strips were assayed for radioactivity with a paper-strip counter. Four radioactive areas were found: one at the origin, which was accompanied by ultraviolet absorption, corresponding to alkylated DNA; two, with R_F values 0·2 and 0·45, corresponding to 7-β-hydroxyethylthioethylguanine and its product of oxidation on the paper (Brookes & Lawley, 1960); and one near the solvent front corresponding to [^{35}S]thiodiglycol resulting from hydrolysis of unreacted 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide. Throughout the experiment the radioactivity at the solvent front was a constant proportion (48%) of the total radioactivity on the paper. However, the radioactivity associated with the 7-alkylguanine products increased, being zero after 30 min. and 42% after 14 days, whereas the radioactivity at the origin decreased correspondingly. The results of the experiment are shown in Fig. 1.

In a similar experiment with the sodium salt of deoxyguanylic acid (39 mg.) and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0·1 mg.) the hydrolysis of the 7-alkyldeoxyguanylic acid to yield 7-β-hydroxyethylthioethylguanine was found to be a first-order reaction with half-life of 8 hr.

Determination of the proportion of combined ^{35}S-labelled mustard gas present as di-(β-guanin-7-ylethyl) sulphide in the reaction of mustard gas with nucleic acids. Nucleic acid (RNA, DNA or DNA denatured by heating at 100° for 10 min. in 0·01 N-phosphate buffer, pH 7·1) (40 mg.) was dissolved in 0·02 N-phosphate buffer, pH 7·1 (10 ml.), at 37°, and ^{35}S-labelled mustard gas (0·35 mg.; 55 μc) in ether (0·01 ml.) was added. After 0·5, 1, 2, 5 and 15 min. samples (2 ml.) were taken, nucleic acid was precipitated with 2 vol. of ethanol containing 2% of sodium acetate cooled to 0°, and dried, and the radioactivity assayed. The samples were hydrolysed with N-HCl (0·5 ml.), and 0·05 ml. was applied to a strip of Whatman no. 1 paper and chromatographed with methanol–conc. HCl–water (7:2:1, by vol.) as solvent. The paper was dried and the radioactivity assayed on a paper-strip counter. Two areas of radioactivity were found, one at the origin corresponding to di-(β-guanin-7-ylethyl) sulphide, and another with R_F 0·1–0·4 corresponding to 7-β-hydroxyethylthioethylguanine and related products of monofunctional alkylation. The ratio of the radioactivity of the former to the total was a measure of the extent of difunctional alkylation, as shown in Fig. 2.

Binding in vivo of 2-hydroxyethyl 2-chloroethyl [^{35}S]-sulphide and of ^{3}H-labelled myleran. Four mice bearing 7-day-old Ehrlich-ascites tumours were each injected intraperitoneally with 2-hydroxyethyl 2-chloroethyl [^{35}S]-sulphide (0·2 mg.; 31 μc) in arachis oil (0·1 ml.). After 1 hr. the mice were killed and RNA, DNA and protein were isolated from the ascites cells as described by Brookes & Lawley (1960). In a similar experiment tumour-bearing mice were injected with ^{3}H-labelled myleran (1 mg.; 3 mc) suspended in arachis oil (0·1 ml.). Mice were killed after 24, 72 and 120 hr.

In another series of experiments C3H mice with advanced virus-induced lymphoblastic leukaemia were used and RNA, DNA and protein were isolated from leukaemic spleen and lymphocytes 22 and 66 hr. after injection of ^{3}H-labelled myleran. The RNA contained 6·8% of P; molar base ratios, adenine 1, guanine 1·75, cytosine 1·58, uracil 0·68. DNA contained 7·1% of P; molar base ratios, adenine 1, guanine 0·79, cytosine 0·72, thymine 0·88, uracil 0·09. The presence of uracil in the DNA shows that RNA was present as an impurity to the extent of about 9%. This results from omission of ribonuclease treatment in order to avoid prolonged dialysis of DNA with consequent loss of alkylated guanine.

Samples of RNA, DNA and protein were assayed for radioactivity (Table 2) and when 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide had been used, radioautographs from chromatograms of hydrolysed nucleic acids were prepared in the usual way.

Tumour growth inhibition by mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide. A C3H mouse bearing the 12-day-old Gardner-ascites tumour was injected with ^{35}S-labelled mustard gas (0·1 mg.; 1 μc) in arachis oil (0·1 ml.), and after 1 hr. the mouse was killed, the ascites cells (3×10^8) were removed and washed with 0·9% NaCl soln., and 10^6 cells injected into each of six C3H mice. A portion of the remainder of the cells was dried and assayed for radioactivity. Similarly, a tumour-bearing mouse was injected with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0·1 mg.; 15·2 μc) and a further six mice were injected with 10^6 mustard-treated ascites cells. A control group of six mice were injected at the same time with the same number of untreated ascites cells. The assay of radioactivity showed that the mustard gas-treated cells contained 5 μg. of bound mustard gas/g. of cells and that the 2-hydroxyethyl 2-chloroethyl sulphide-treated contained 4 μg. of bound 2-hydroxyethyl 2-chloroethyl sulphide/g. The mice were examined at intervals. After 8 days the control and 2-hydroxyethyl 2-chloroethyl sulphide-treated groups had developed tumours, whereas the mustard gas-treated group had no visible tumour growth. The numbers of survivors after 28 days were: controls, 0; 2-hydroxyethyl 2-chloroethyl sulphide-treated, 1; mustard gas-treated, 6.

In similar experiments doses of 30 μg. of mustard gas and 1 mg. of 2-hydroxyethyl 2-chloroethyl sulphide were used. Again the mustard gas-treated cells did not yield tumours on reinjection, whereas the 2-hydroxyethyl 2-chloroethyl sulphide-treated gave visible tumours within 14 days.

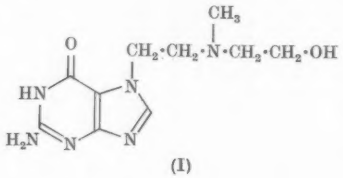
Table 1. *Reaction of alkylating agents with nucleic acids at* pH 7 *and* 37°

Concn. of nucleic acid (M)	Concn. of reagent (M)	Concn. of buffer (M)	Half-life of reagent (hr.)		Extent of reaction as % of reagent combined with nucleic acid	Product(s)
			Reaction with nucleic acid	Hydrolysis in water		
RNA (yeast), 0·05	Methyl methanesulphonate, 0·05	Phosphate, 0·4	—	9·2	1·9	7-Methylguanine
RNA (yeast), 0·05	Ethyl iodide, 0·05	Phosphate, 0·4	—	—	1·1	7-Ethylguanine
RNA (yeast), 0·05	Di-(2-chloroethyl)methylamine, 0·025	Phosphate, 0·4	—	2·2	18	β-(Guanin-7-yl)ethyl-β-hydroxyethylmethylamine and di-(β-guanin-7-ylethyl)methylamine
RNA (yeast), 0·1	Methyl methanesulphonate, 0·1	Acetate, 0·4	—	—	5·7	7-Methylguanine
RNA (yeast), 0·1	Ethyl methanesulphonate, 0·1	Acetate, 0·4	—	10·4	1·8	7-Ethylguanine
RNA (rat liver), 0·1	[^{14}C]Ethyl iodide, 0·002	Phosphate, 0·01	~40	—	0·95	7-Ethylguanine
RNA (rat liver), 0·05	[^{14}C]Ethyl methanesulphonate, 0·01	Phosphate, 0·05	10	—	0·75	7-Ethylguanine
RNA (rat liver), 0·05	^{14}C-Labelled myleran, 0·008	Phosphate, 0·03	9	2	1·8	7-δ-Hydroxybutylguanine and αδ-di-(guanin-7-yl)butane
RNA (rat liver), 0·067	*NN*-Di-(β-chloroethyl)[^{14}C]-aniline, 0·0024	Acetate, 0·02, 50% ethanol	8	—	65	See text
RNA (rat liver), 0·01	Di-(2-chloro[^{14}C]ethyl)amine, 0·0002	Phosphate, 0·002	10	—	67	See text
RNA (rat liver), 0·01, or DNA, 0·01	^{35}S-Labelled mustard gas, 0·0001	Phosphate, 0·001	0·02	—	80	7-β-Hydroxyethylthioethyl-guanine and di-(β-guanin-7-ylethyl) sulphide
RNA (rat liver), 0·01, or DNA, 0·01	2-Hydroxyethyl 2-chloroethyl [^{35}S]sulphide, 0·0001	Phosphate, 0·001	0·02	0·02	75	7-β-Hydroxyethylthioethyl-guanine
DNA, 0·1	Ethyl sulphate, 0·1	Acetate, 0·4	—	—	0·7	7-Ethylguanine
DNA, 0·1	^{14}C-Labelled myleran, 0·005	Acetate, 0·05	—	—	0·7	7-δ-Hydroxybutylguanine and αδ-di(guanin-7-yl)butane
DNA, 0·01	[^{14}C]Ethyl methanesulphonate, 0·0025	Phosphate, 0·02	~12	—	0·4	7-Ethylguanine
RNA, 0·01	[^{14}C]Ethyl methanesulphonate, 0·0025	Phosphate, 0·02	~12	—	0·8	7-Ethylguanine
DNA, 0·01	[^{14}C]Methyl methanesulphonate, 0·0025	Phosphate, 0·02	8	—	4	7-Methylguanine
RNA, 0·01	[^{14}C]Methyl methanesulphonate, 0·0025	Phosphate, 0·02	8	—	8	7-Methylguanine

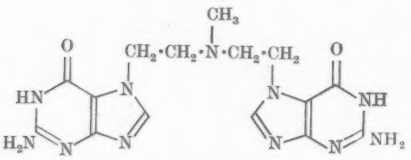
RESULTS

The alkylation of nucleic acids in neutral aqueous solution by a variety of agents has been shown, as expected, to proceed at a rate approximately equal to the rate of hydrolysis of the alkylating agents in water (Table 1). The extent of reaction with nucleic acids was not necessarily proportional to the rate of reaction, e.g. with di-(2-chloroethyl)amine and *NN*-di-(2-chloroethyl)-aniline the major part of the reagent reacted with nucleic acid, whereas with ethyl methanesulphonate and myleran the extent of reaction with nucleic acid was small, although in all four cases the rate of reaction was approximately the same.

The site of alkylation of the nucleic acids was shown in all cases to be N-7 of guanine moieties by comparison of the products isolated from the acid-hydrolysed alkylated nucleic acids with those from the alkylation of guanosine or guanylic acid (Brookes & Lawley, 1961*b*). The difunctional agents gave in all cases two types of product. The first type had R_F values greater than those for guanine, i.e. were 7-alkylguanines, e.g. (I), resulting from reaction of one alkylating group with a guanine moiety and the second with water. The second type had zero R_F values, characteristic of di-(guanin-7-yl) derivatives e.g. (II) resulting from reaction of both alkylating groups with guanine moieties.



(I)



(II)

With *NN*-di-(2-chloroethyl)aniline the products from guanosine were rapidly oxidized in air and could not be analysed. With di-(2-chloroethyl)-amine the R_F values of the products were closely similar to the analogous products from HN 2 [di-(2-chloroethyl)methylamine] and their structures have been assigned on that basis as β-(guanin-7-yl)-ethyl-β-hydroxyethylamine and di-(β-guanin-7-ylethyl)amine.

It had been shown (Lawley, 1957; Brookes & Lawley, 1961*b*) that after alkylation of deoxyguanylic acid the product was hydrolysed at neutral pH yielding 7-alkylguanine. In the present work alkylated DNA hydrolyses in a similar way. In the alkylation of DNA with labelled alkylating agents the specific radioactivity of DNA increased initially at the same rate as for RNA, but subsequently decreased, the lost radioactivity being found in the supernatant after precipitation of DNA. This radioactivity was shown to be associated with 7-alkylguanines and di-(guanin-7-yl) derivatives resulting from hydrolysis of the alkylated DNA.

In a more detailed study of the time course of the hydrolysis of DNA at pH 7 and 37° after its alkylation by 2-hydroxyethyl 2-chloroethyl [^{35}S]-sulphide (Fig. 1) the initial rate of hydrolysis was about the same as for the corresponding 7-alkyl-deoxyguanylic acid, but subsequently the rate decreased, the overall half-life being about 52 hr. After 300 hr. less than 10% of the alkyl groups remained attached to DNA. This residual alkylated DNA was hydrolysed and the products were identical with those initially found.

Comparison of the reaction with nucleic acids of mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide showed that the only difference was the absence of formation of di-(β-guanin-7-ylethyl) sulphide in the latter case. This was also the only difference observed for the reaction of the reagents with nucleic acids *in vivo*, although the difunctional agent was shown to be at least 30 times as effective as the monofunctional in inhibiting the growth of an ascites tumour.

In a comparison of these mustards with myleran, with regard to their binding to cellular constituents

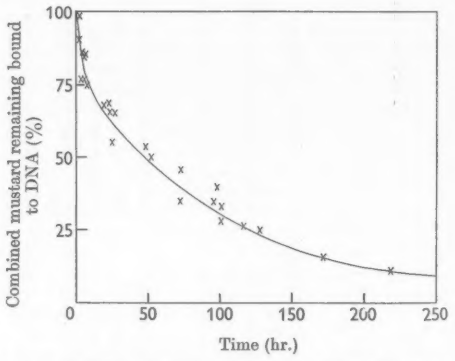


Fig. 1. Hydrolysis in 0·01 N-phosphate buffer, pH 7·1t, a 37°, of DNA alkylated with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0·01 mole/mole of DNA P).

Table 2. *Amount of reaction of* ^{35}S*-labelled mustard gas, 2-hydroxyethyl 2-chloroethyl* [^{35}S]*sulphide and* ^{3}H*-labelled myleran with cellular constituents in the mouse*

Mice bearing the Ehrlich-ascites tumour or with virus-induced lymphoblastic leukaemia were injected intraperitoneally with the reagent in arachis oil and killed after the time stated. Cellular constituents were isolated from ascites or leukaemic cells.

Type of cell	Reagent	Dose (mg./kg.)	Dose (μc/g.)	Time (hr.)	Specific radioactivity (μc/g.) Protein Cytoplasm	Protein Nucleus	RNA	DNA	Amount of reaction with nucleic acid (μmoles/mole of nucleic acid P) RNA	DNA
Ascites	^{35}S-Labelled mustard gas	2	0·32	0·5	1·9	1·9	2·4	2·2	40	37
Ascites	2-Hydroxyethyl 2-chloroethyl [^{35}S]sulphide	4	0·63	1	2·9	2·6	4·0	3·6	75	70
Ascites	^{3}H-Labelled myleran	20	60	24	10·6	7·1	2·1	4·3	1·2	2·4
Ascites	^{3}H-Labelled myleran	20	60	72	5·6	4·0	2·2	4·2	1·2	2·3
Leukaemic	^{3}H-Labelled myleran	40	120	22	3·3	1·6	5·3	6·4	3·3	3·7
Leukaemic	^{3}H-Labelled myleran	40	120	66	1·9	1·7	4·0	4·0	2·2	2·2

Fig. 2. Proportion of combined mustard gas present as di-(β-guanin-7-ylethyl) sulphide in the reaction of nucleic acids with ^{35}S-labelled mustard gas: m-mole of mustard gas combined/mole of nucleic acid P after 15 min.: ×, DNA, 1·6; +, DNA, 6·3; △, heat-denatured DNA, 1·4; ○, RNA, 2·9; ●, RNA, 16.

in vivo (Table 2), it was found that the mustards were bound to an equal extent, which was about 20 times that found for myleran given at a tenfold higher dose. A similar low extent of binding of myleran to leukaemic cells was found.

Reaction of difunctional alkylating agents with both RNA and DNA was shown to yield a proportion of di(guanin-7-yl) derivatives. Studies of the rate of formation of di-(β-guanin-7-ylethyl) sulphide (Fig. 2) showed that as expected it was less than that of the primary alkylation reaction. The proportion of combined mustard gas present as di-(β-guanin-7-ylethyl) sulphide was greatest for DNA, less for heat-denatured DNA and least for RNA (Fig. 2).

DISCUSSION

Comparisons of the rates and extents of alkylation of nucleic acids by a variety of alkylating agents have shown that in general with the mustard type of reagent a high extent of alkylation is attained, although the rates of reaction varied from half-lives of a few minutes to several hours (Table 1). On the other hand, the methanesulphonate, sulphate or halide type of reagent reacted to a much lower extent. This difference was also found when the binding to cellular constituents *in vivo* of mustard gas or of 2-hydroxyethyl 2-chloroethyl sulphide was compared with that of myleran (Table 2).

The finding reported by Brookes & Lawley (1960) that the sole site of alkylation of nucleic acids by mustard gas when the extent of reaction is

small, is at N-7 of guanine moieties, has now been confirmed for a number of alkylating agents. It has also been established that with the difunctional alkylating agents used products are formed by linkage of two guanine moieties by alkyl chains, e.g. (II).

Myleran, mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide were chosen for studies *in vivo* since they represent respectively weakly reactive difunctional, highly reactive difunctional and highly reactive monofunctional alkylating agents. In tests against the Ehrlich-ascites tumour in the mouse, myleran was inactive at a dose of 20 mg./kg./day (Sugiura, 1956), and the present work has shown that 2-hydroxyethyl 2-chloroethyl sulphide is inactive at a similar dose level, whereas mustard gas is active at one-thirtieth of this dose. The low anti-tumour activity of myleran is perhaps expected in view of the low level of binding to the cellular constituents found (Table 2). The present findings do not suggest any explanation of the effectiveness of myleran against chronic myelogenous leukaemia (Haddow & Timmis, 1953).

Consideration of the overall extent of binding to cellular constituents clearly cannot explain the marked difference in cytotoxic activity found for the mono- and di-functional sulphur mustards. The explanation must therefore be sought in differences in the mode of binding of these reagents. With nucleic acids both mustards react rapidly and to the same extent to give identical products, except that the difunctional reagent yields a proportion of a cross-linked guanine product. If the biological activity of these agents is to be ascribed to reaction with nucleic acid then the formation of this type of product must be of importance, and it is clearly of interest to attempt to determine the relative dispositions of the linked guanine moieties in the nucleic acid structure.

The model proposed by Crick & Watson (1954) for the structure of DNA shows that the N-7 atoms of guanine moieties are sterically well available for reaction. For a pair of these atoms to be situated at a distance apart such that they could be linked by an extended alkyl chain of 4 or 5 atoms, i.e. about 8 Å, it appears from this model that the sequence of bases along the DNA macromolecular chain must be guanine–cytosine along one strand (and by the necessary complementary pairing of bases, cytosine–guanine on the other strand), and in one direction only, namely deoxyguanylyl-(3′ → 5′)-deoxycytidylyl. With this sequence guanine moieties on each of the twin strands could be readily cross-linked, as represented diagrammatically in Fig. 3. If the order of the guanine–cytosine sequence is reversed the cross-linking of the guanine moieties is no longer possible. Linkage of adjacent guanine moieties on the same molecular strand of DNA cannot be eliminated on steric grounds, but would require the alkyl chain to assume a less probable non-extended configuration.

Support for the model of cross-linking represented in Fig. 3 comes from consideration of the proportion of cross-linked guanine resulting from alkylation of DNA, denatured DNA and RNA (Fig. 2). Whereas DNA yields 26% of the total alkylation products in this form, the proportion becomes progressively less in passing to denatured DNA and RNA, in spite of the higher proportion of guanine in RNA. However, this is the order of decreasing extent of twin-stranded structure of the nucleic acids according to the Crick–Watson model, and decreasing yield of a product derived from such a structure would therefore be expected.

The greater cytotoxic effect of the difunctional alkylating agents could clearly be explained by accepting this model of cross-linking of the twin strands of DNA. According to the Crick–Watson model separation of these twin strands is necessary for cell division and this would be inhibited if the strands were joined through a covalent bond as proposed. Further, the loss of 7-alkylguanine from DNA, which has been shown to follow its alkylation, would lead to fission of the sugar phosphate chain (Brown & Todd, 1955), but breaking of the twin-stranded DNA molecule would be expected

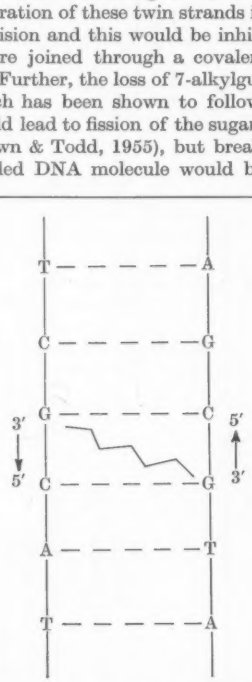


Fig. 3. Diagrammatic representation of cross-linking by a difunctional alkylating agent of the twin strands of DNA according to the model of Crick & Watson. The order of bases along the molecular chain is shown by their initial letters; dotted lines represent the hydrogen bonds between the bases; the alkyl chain is indicated by the zig-zag line; the required sequence of bases is deoxyguanylyl-(3′→5′)-deoxycytidylyl along either chain.

only if alkylation on each strand at nearly opposite points had occurred. At low degrees of alkylation by monofunctional agents this would occur only rarely, being in proportion to the square of the degree of alkylation; with difunctional agents about a quarter of the alkylations would be of this type. It would also be expected that fission of the DNA molecule could seriously interfere with its biological function.

SUMMARY

1. The rates and extents of alkylation of ribonucleic acid and of deoxyribonucleic acid in neutral aqueous solution at 37° by a variety of alkylating agents have been determined.

2. Alkylation has been shown to occur at N-7 of guanine moieties, monofunctional agents yielding 7-alkylguanines, and difunctional agents yielding in addition di(guanin-7-yl) derivatives.

3. Alkylated ribonucleic acid is stable in neutral aqueous solution but alkylated deoxyribonucleic acid decomposes with loss of the alkylated guanine products.

4. The extent of binding has been determined of ^{3}H-labelled myleran, ^{35}S-labelled mustard gas and of 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide to cellular constituents of the Ehrlich-ascites tumour and of ^{3}H-labelled myleran to leukaemic cells in the mouse.

5. The only difference found *in vivo* or *in vitro* for reaction of mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide with nucleic acids is that mustard gas yields di-(β-guanin-7-yl) sulphide.

6. Mustard gas is at least 30 times as effective as 2-hydroxyethyl 2-chloroethyl sulphide as an inhibitor of the growth of an ascites tumour in the mouse.

7. The mode of combination of monofunctional and of difunctional alkylating agents with nucleic acids is discussed in terms of the Crick–Watson model for the structure of deoxyribonucleic acid.

8. The possible relationship between the alkylation of deoxyribonucleic acid and the biological properties of the alkylating agents is discussed.

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The Relative Stabilities of the Skeletal-Muscle Myosins of some Animals

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It is sometimes found that the stabilities of the members of a series of closely related proteins differ widely, examples being the haemoglobins and the collagens. It has been proposed (Haurowitz, Hardin & Dicks, 1954) that the relative stabilities towards alkaline denaturation of the haemoglobins of various animals are due to different degrees of complementariness of the globin molecules to the haem group. Differences in the hydrothermal stability of collagens from certain animals

have been ascribed to differences in hydroxyproline content (Gustavson, 1955; Rigby & Spikes, 1960). There appears to be a correlation between the hydrothermal stability of fish-skin collagen and the environmental temperature of the fish from which the collagen was prepared.

The myosins of different species form another such series of closely related proteins. It has been shown that the molecular parameters and amino acid composition of the myosins of rabbit and cod skeletal muscle are very similar (Connell, 1958, 1959), and other, fragmentary, evidence in the literature suggests that in these respects the skeletal-muscle myosins of other animals would be similar. Nevertheless, there are indications that the myosins of different species vary appreciably in their stability. Thus cod myosin denatures and aggregates much more readily than rabbit myosin (Connell, 1960). Frog (Hamoir & Reuter, 1956) and carp (Hamoir, McKenzie & Smith, 1960) myosins are reported to be unstable but mullet myosin is said to be stable (Hamoir *et al.* 1960).

This paper is concerned with extending these observations on relative stability in a more systematic fashion. Different preparations of myosin from the same type of muscle vary somewhat in their apparent stability and it was therefore deemed advisable when comparing the stabilities of myosin from different types of muscle to use conditions as nearly the same as possible from preparation to preparation and to employ several criteria of stability. The criteria used are: (i) measurement of the rate of aggregation [which really measures the rate of heat denaturation (Connell, 1960)]; (ii) the effect of increasing concentrations of urea on optical rotation, reactive sulphydryl groups and adenosine triphosphatase activity; (iii) rate of digestion by trypsin.

EXPERIMENTAL

Materials

Skeletal muscle from the following animals has been used: ox, rabbit, chicken, frog (*Rana temporaria* L.), cod (*Gadus callarias* L.), haddock (*G. aeglefinus* L.), lemon sole (*Pleuronectes microcephalus* Day), plaice (*P. platessa* L.), turbot (*Rhombus maximus* Day), herring (*Clupea harengus* L.) and halibut (*Hippoglossus vulgaris* Day). All the fishes, with the exception of the cod, were killed and gutted at sea and stored for a few days in melting ice before use. In all other cases, the muscle was removed from the animal before the onset of rigor. Occasionally the muscles were rapidly frozen in an air-blast freezer, wrapped in aluminium foil and stored for a few days at $-30°$ before use. The anatomical locations of the muscle used were respectively: frog: leg muscles; chicken: breast muscle; fishes: in all cases the skeletal muscle from both sides of the body.

Myosins were prepared from these muscles by the method described for cod muscle (Connell, 1960). Muscle from individual animals has been used for each preparation, except with frog, where muscle from five animals was pooled.

Methods

First-order rates of aggregation of the fish myosins were measured (Connell, 1960) from ultracentrifuge diagrams of solutions of the myosins stored at 0° in 0·45 M-KCl–3·38 mM-KH_2PO_4–15·5 mM-Na_2HPO_4 (total I 0·5), pH 7·5, protein concentration 0·4–0·5%. Rates of aggregation of the other myosins stored under the same conditions were not measured quantitatively.

The methods for the determination of optical rotation, sulphydryl groups, adenosine triphosphatase activity and tryptic digestion have been described (Connell, 1960). Care was taken to treat the myosins in an exactly parallel fashion. With solutions used for the determination of enzymic activity, urea was added just before the assay. Removal of the urea by dialysis before performing the enzyme assay did not materially affect the results. In all other cases, solutions made up to different concentrations of urea were stored at 0° overnight and then clarified by centrifuging at 100 000g for 45 min. In any one series of myosin solutions containing different concentrations of urea, the protein concentrations were the same, though not necessarily the same for different series.

RESULTS

First-order rates of aggregation (k) for the fish myosins are shown in Table 1. Results on cod, haddock, lemon sole and herring were obtained on six, three, three and two different myosin preparations of each species respectively; those on turbot and plaice were obtained on one preparation only. The values of k were usually calculated from the amount of monomeric myosin remaining after 2, 3, 5 and 6 days' storage. The low rate of aggregation of turbot myosin with respect to the other fish myosins has been confirmed qualitatively with another preparation. The behaviour of halibut myosin could not be properly assessed because even freshly prepared samples showed in the ultracentrifuge an apparently polydisperse component sedimenting slightly faster than, and partly obscuring, the main (presumably) myosin peak. As far as could be judged from the rate of disappearance of the visible part of the main peak, the

Table 1. *First-order rates of aggregation* (k) *of fish myosins at* 0° (pH 7·5, I 0·5)

Species from which myosin was prepared	10^5 k sec.$^{-1}$
Haddock	0·23 (0·05*)
Cod	0·20 (0·06*)
Plaice	0·19
Lemon sole	0·11 (0·03*)
Turbot	0·07
Herring	0·03

* Standard deviation.

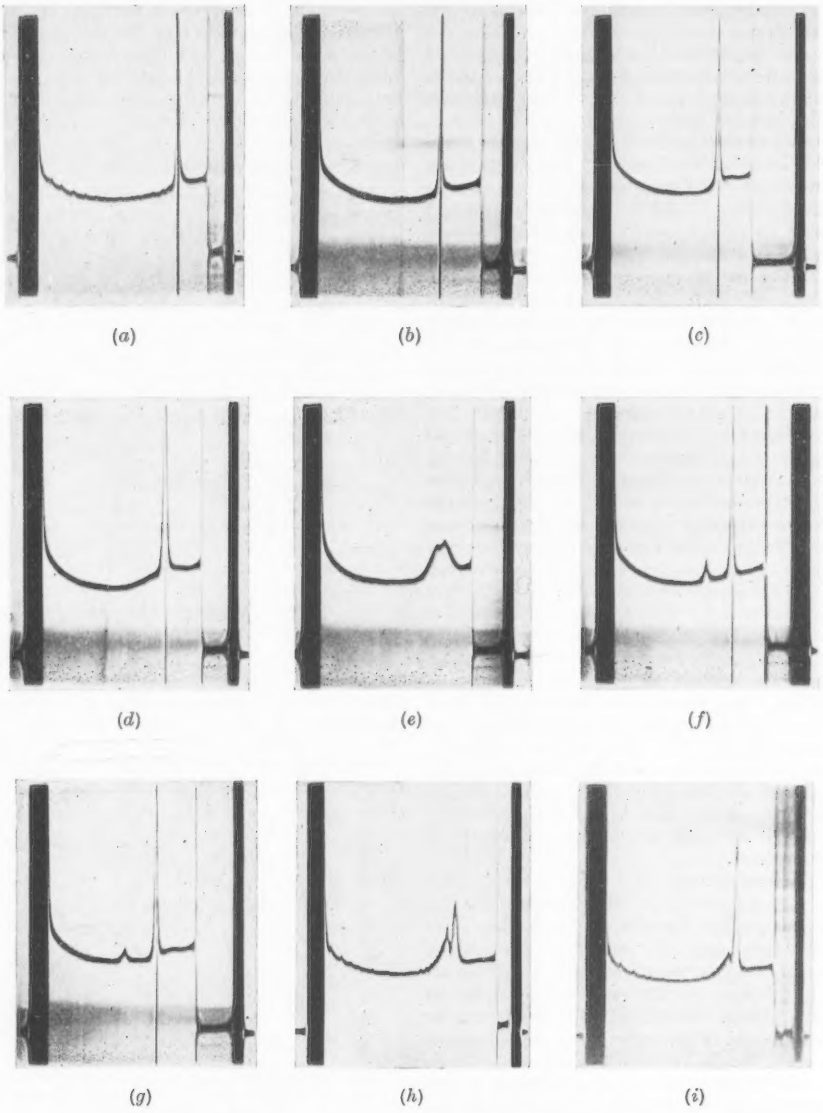

Fig. 1. Ultracentrifuge diagrams of various myosins during storage at 0°, pH 7·5, *I* 0·5. (*a*) Rabbit myosin [protein concentration (*c*) 0·95 %] after 7 days; (*b*) and (*c*) ox myosin (*c*, 1·23 %) after 7 and 10 days respectively; (*d*) and (*e*) frog myosin (*c*, 1·57 %) after 7 and 10 days respectively; (*f*) and (*g*) chicken myosin (*c*, 0·57 %) after 7 and 10 days respectively; (*h*) cod myosin (*c*, 0·43 %) after 6 days; (i) lemon sole myosin (*c*, 0·54 %) after 6 days. Sedimentation from right to left, 69 min. after reaching full speed of 59 780 rev./min., with the exception of (*a*), (*h*) and (*i*), where the speed is 56 100 rev./min. and (*c*) where the time is 74 min. after reaching full speed. Temperature of runs: (*a*) 1·9°; (*b*) 5·9°; (*c*) 3·9°; (*d*) 5·8°; (*e*) 3·9°; (*f*) 3·2°; (*g*) 2·9°; (*h*) 4·0°; (*i*) 2·6°.

preparations of halibut 'myosin' were of about average stability with respect to the other fish myosins.

The rate of aggregation of cod myosin measured in the present fuller series of experiments is somewhat higher than, but not out of line with, that quoted by Connell (1960).

Fig. 1 shows the results of storage of other myosins. All the solutions of myosins other than those from fishes and frog were stored at protein concentrations of about 1·0–1·5 %. Aggregation (of cod myosin at least) is favoured at such high protein concentrations (Connell, 1960). Three preparations each of rabbit and ox myosins have been examined and in no case were aggregates visible after storage for 7–10 days under these conditions, though slight broadening of the ox-myosin boundary is discernible after 10 days' storage. Only one preparation each of chicken and frog myosins was examined. The former was stable up to 10 days, but the ultracentrifuge diagram of the latter shows broadening of the myosin boundary after 7 days' storage and the definite presence of aggregates after 10 days. The small component in the chicken-myosin diagram (Fig. 1*f* and *g*) sedimenting more rapidly than the main component is presumably actomyosin. The appearances in the ultracentrifuge of cod and lemon sole myosins stored for 6 days are included in Fig. 1 for comparison.

It is clear that when myosins from different species are stored under identical conditions there are marked, consistent differences in their rates of aggregation. Fish myosins as a whole are more unstable in this respect than those of other species. Within the fish species an order of relative stability emerges, herring myosin being the most stable and cod, haddock and plaice myosins the least. By this criterion frog myosin is more stable than the fish myosins but not quite as stable as ox, rabbit or chicken myosins.

The myosins can also be divided into two main groups differing in their behaviour towards increasing concentrations of urea (Figs. 2–4). With ox, rabbit and chicken myosins only very small or no changes occur in optical rotation, freely titratable sulphydryl groups or adenosine triphosphatase activity until a concentration of 2–3 M-urea is reached, whereas all the fish myosins already show appreciable change at concentrations of 0·5–1·0 M-urea. Bárány, Bárány & Trautwein (1960) have recently shown that the adenosine triphosphatase activity of rabbit-myosin solutions activated by calcium is virtually unchanged in concentrations of urea up to 2 M. Steven & Tristram (1959) studied the effect of increasing concentrations of urea on various properties of ovalbumin and found evidence for the suggestion (Schellman, 1955) that there is a critical urea concentration for each protein at which the rate of denaturation is accelerated. It would seem that this critical urea concentration is very low for fish myosins and about 2–3 M for ox, rabbit and chicken myosins. Very low concentrations of urea are also effective in releasing a protein fraction of low molecular weight from cod myosin, whereas concentrations of between 2 and 3 M-urea are necessary to effect release of similar material from rabbit or ox myosin

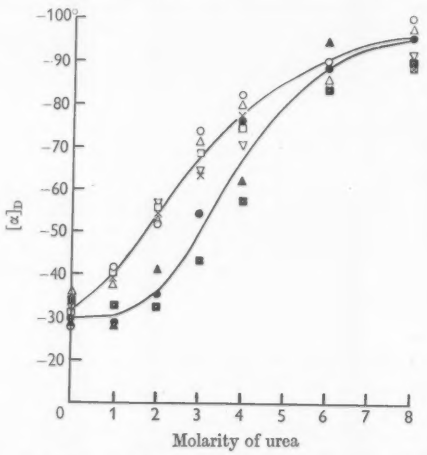

Fig. 2. Effect of different concentrations of urea on the optical rotation of various myosins. ●, Rabbit; ■, ox; ▲, chicken; ○, cod; □, haddock; △, lemon sole; ×, plaice; ▽, halibut. For the sake of clarity some of the experimental points have been omitted.

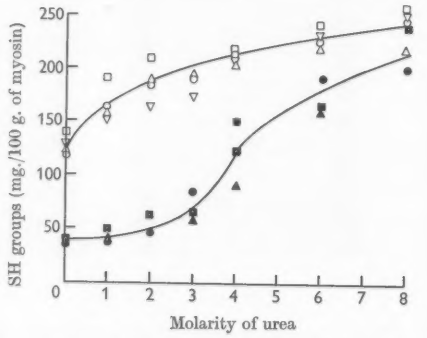

Fig. 3. Effect of different concentrations of urea on the freely titratable sulphydryl groups of various myosins. ●, Rabbit (3); ■, ox (3); ▲, chicken (1); ○, cod (2); □, haddock (2); △, lemon sole (2); ▽, halibut (2). The experimental points are the mean results of the numbers of determinations given in parentheses.

(Connell, 1960; J. J. Connell & H. S. Olcott, unpublished work).

The numbers of sulphydryl groups titratable with *p*-chloromercuribenzoate in the native fish myosins are about double those in the more stable myosins, though the total numbers of sulphydryl groups in all the myosins are approximately the same. This is the only chemical difference that has so far been found between unstable and stable myosins. It may be connected with the fact that chemical reaction of the sulphydryl groups of cod myosin results in a more rapid aggregation of the protein (Connell, 1960). Tsao & Bailey (1953) found that about half the total sulphydryl groups of rabbit myosin are titratable with *N*-ethylmaleimide.

The results of tryptic digestion of the myosins at 25° are shown in Fig. 5. The amounts of hydrolysis are indicated by the extinctions of ninhydrin-treated samples of the digests after removal of protein by precipitation with trichloroacetic acid (Connell, 1960). From their rates of digestion the myosins again fall into two distinct groups, the members of each of which behave very similarly. The fish myosins form one group, which in the initial stages is digested much more quickly than the other group, comprising ox, rabbit and chicken myosins. The same division is also evident when the digestions are carried out at 15° and 40°. In all cases the rate curves can be approximately fitted to the sum of two first-order-reaction curves (Harrington & Mihalyi, 1959; Connell, 1960). Considering the overall digestion reaction to be the result of two parallel first-order digestion reactions of different rate, the proportion of bonds split by the fast reaction is about 50% for the fish myosins and about 15–25% for the ox, rabbit and chicken myosins. The latter figure agrees with that found by Harrington & Mihalyi (1959) for the tryptic digestion of rabbit myosin at a similar temperature.

The course of the tryptic digestion of cod myosin has also been followed in some preliminary ultracentrifuge experiments by arresting the reaction at intervals with soyabean trypsin inhibitor. Digestion of cod myosin at pH 7·5 resembled that of rabbit myosin at pH 8·9 (Harrington & Mihalyi, 1959) in that at no stage was there liberation of two completely separate components corresponding to L- and H-meromyosins. A rapid liberation of a well-defined slow component occurred but at least

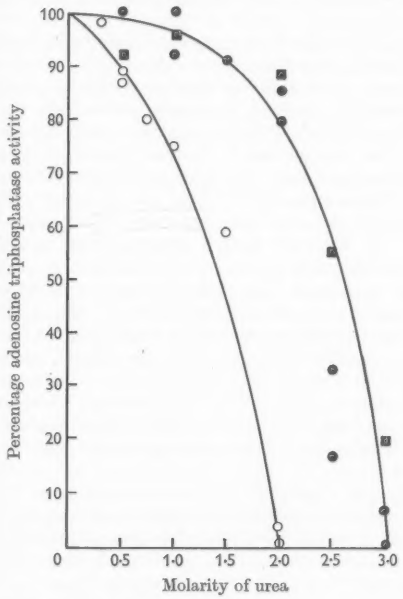

Fig. 4. Effect of different concentrations of urea on the adenosine triphosphatase activity of various myosins. ■, Ox (1); ●, chicken (2); ○, cod (2). Activities are expressed as a percentage of the activities of the individual preparations in the absence of urea. The experimental points are the mean results of the numbers of determinations given in parentheses.

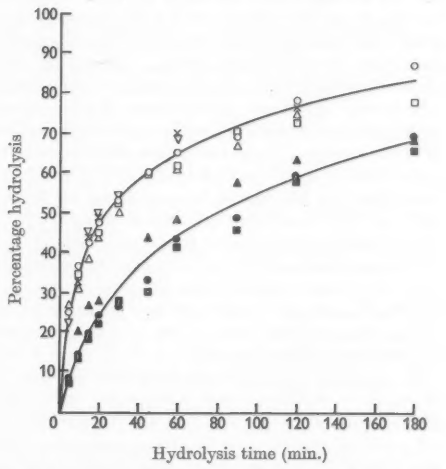

Fig. 5. Tryptic digestion of various myosins at 25°, pH 7·5, *I* 0·5. ●, Rabbit (2); ■, ox (2); ▲, chicken (1); ○, cod (5); □, haddock (1); △, lemon sole (2); ▽, halibut (1); ×, turbot (2). Amounts of hydrolysis at given times are expressed as percentages of the amounts of hydrolysis of individual preparations after 24 hr. digestion. The experimental points are the mean results of the numbers of determinations given in parentheses. Some of the experimental points up to 20 min. digestion have been omitted for the sake of clarity.

two polydisperse faster components were present at all stages of the digestion. Hydrolysis of cod myosin for 1 min. at pH 7·5 and 17° gave an ultracentrifuge diagram which had approximately the appearance of that of rabbit myosin hydrolysed for 20 min. at pH 8·1 and 25°. Digestion of cod myosin at pH 6·5 and 0° was also extremely rapid, splitting of the protein into two major and one minor ultracentrifuge components being complete within 1 min.

DISCUSSION

By all the criteria used the myosins examined, with the exception of frog myosin, fall into two distinct stability groups, the group of fish myosins being more unstable than the group comprising ox, rabbit and chicken myosins. Frog myosin (judged from its rate of aggregation alone) is somewhat less stable than the latter group. These observations suggest that the stability of myosins is related to biological function or environment. Support for this view is available from two sources. In the first, Mirsky (1938) found that frog actomyosin was more easily heat denatured than rabbit actomyosin, and in the second, Braun, Nesvetaeva & Fizhenko (1959) found that the actomyosins of grass and lake frogs differ in their resistance to the denaturing effects of alcohol and heat. The fact that frog myosin is not as unstable as the fish myosins suggests that stability is not entirely, if at all, a consequence of cold-bloodedness. However, the present results do indicate that as far as stability is concerned, myosins of different species have become adapted to the different body temperatures of these species. Thus the fishes studied in this paper live at temperatures of about 10°, the frogs were kept at laboratory temperatures of 15–20° and the body temperatures of the other animals will be in the physiological range of about 39–41°. In this connexion it may not be without significance that the only really stable fish myosin so far described, namely mullet myosin (Hamoir *et al.* 1960), was prepared from a fish inhabiting warm sub-tropical surface waters.

In general, proteins susceptible to one set of denaturing conditions are susceptible to others. This consideration can account for the parallelism between the susceptibilities of the myosins to the denaturing effects of urea and their rates of aggregation, the latter being tantamount to rates of heat denaturation. However, it seems that aggregation is a more sensitive guide to relative stability than is behaviour on urea denaturation: the rates of aggregation of the fish myosins differ measurably from one another but their behaviour in urea is very similar.

It has been proposed (Connell, 1960) that cod muscle is more easily damaged by processes like freezing or dehydration than are mammalian muscles because cod myosin is intrinsically more unstable than mammalian myosins. The results in this paper extend this proposal to the myosins of other species. For example, fish muscle in general is more readily damaged than mammalian muscle and it has now been found that the myosins of all the fish species studied are more unstable than those of ox or rabbit. What is more, within the fish species there are variations in the ability of their muscles to withstand freezing and frozen storage, and these variations are also reflected in some degree by corresponding variations in myosin stability. Dyer (1951) and Dyer & Morton (1956) have shown that, judged by changes in the solubility of the myofibrillar proteins and by textural changes of the flesh, halibut muscle withstands frozen storage under standard conditions better than plaice muscle and the latter better than cod muscle. It also is the experience of this laboratory that in this respect lemon sole, halibut and turbot muscle are better than cod or haddock muscle; haddock muscle is possibly better than cod muscle. These relationships have a rough correlation with the relative order of stability of the isolated myosins from these species.

Since the mechanisms involved in denaturation by freezing and frozen storage are likely to be different from that by heat an exact correlation cannot be expected, especially where only small differences in stability exist. In addition, there may be factors other than the stability of the myosins *per se* which influence the stability of the myofibrillar proteins *in situ*, for example, differences in the chemical composition of the muscle. Indeed, Dyer & Morton (1956), Dyer, Morton, Fraser & Bligh (1956) and Dyer & Fraser (1959) have suggested that variations in the behaviour shown by muscles from different fish species towards frozen storage is a consequence of two factors which differ between the species, namely the lipid content of the flesh and the production of free fatty acid during frozen storage. However, further work from this laboratory (Olley & Lovern, 1960) has shown that this viewpoint may need modifying.

At first sight it would seem possible to account for the more rapid tryptic digestion of unstable myosins on the grounds that in general proteolytic enzymes digest denatured more rapidly than native proteins. Thus under similar conditions solutions of unstable myosins will contain more denatured, trypsin-susceptible molecules than those of stable myosins. However, this explanation seems unlikely in view of the fact that heat-denatured cod myosin is digested at the same rate as the native protein (Connell, 1960). Alternative explanations could be (i) that the possible capacity of trypsin to de-

nature (for review see Green & Neurath, 1954) is greater for the unstable myosins, or (ii) that trypsin attacks unstable and stable native myosin molecules equally rapidly but that once a few crucial bonds are broken the former type of molecule unfolds more readily and is ultimately digested more rapidly.

In terms of Harrington & Mihalyi's (1959) interpretation of the rate of tryptic hydrolysis of fibrous proteins like myosin, the more rapid digestion of the unstable myosins means that these proteins possess a larger proportion of 'amorphous' or 'disordered' structure than do their stable counterparts. This follows from the larger proportion in the unstable myosins of bonds split by the 'fast' reaction. However, the similarity in certain structural features between unstable and stable myosins argues against this proposal. For example, the optical rotations, proline contents and overall molecular dimensions of cod and rabbit myosins are the same, suggesting that the amount of 'order' in these two proteins is likely to be similar.

The reasons why proteins denature at different rates presumably depend upon such factors as differences in (i) the numbers and kinds of intramolecular bonds (such as those considered by Kauzman, 1959), (ii) degree of order (for example, amount of helical structure), (iii) degree of compactness, and (iv) goodness of fit of different regions of the molecules. In view of the similarities between different myosins it seems likely that some of these factors, for example degree of order, will be of minor or no significance in determining the relative stability of this series of proteins. In both cod and rabbit myosins the total sulphydryl groups titratable with *N*-ethylmaleimide or *p*-chloromercuribenzoate equal those expected from the cysteine content, that is, intramolecular disulphide bonds are absent and cannot therefore influence the stability of the two proteins.

SUMMARY

1. Myosins from the skeletal muscle of various animals have been prepared and their stabilities compared by a number of methods.

2. By all the criteria of stability used, the myosins mostly fall into two distinct groups: an unstable group comprising all the fish myosins, and a stable group including rabbit, ox and chicken myosins. Frog myosin appears to be somewhat more unstable than the latter group.

3. These results indicate: (i) that, as far as stability is concerned, myosins of different species have become adapted to the different body temperatures of these species; (ii) that there is a connexion between the resistance of a muscle towards such damaging effects as freezing, frozen storage and dehydration, and the stability of the myosin when isolated from it.

Mr P. F. Howgate assisted in the experiments described in this paper. The work described was carried out as part of the programme of the Department of Scientific and Industrial Research.

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Studies on the Phosphoproteins of Brain

3. PHOSPHORYLSERINE SEQUENCES IN BRAIN PHOSPHOPROTEIN*

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In a previous paper it was reported that a phosphoprotein fraction isolated from brain was capable of accepting radioactive phosphate in a suitable phosphorylating system and that the phosphate incorporated could subsequently be isolated as phosphorylserine (Heald, 1961*a*). It was later shown (Rose & Heald, 1961) that this phosphoprotein fraction was attacked, to a limited degree, by a specific phosphoprotein phosphatase isolated from brain.

These properties are shared with the phosphoproteins casein and phosvitin, which have been shown to contain sequences of phosphorylserine residues of the type $(\text{Ser-P})_n$, where n can range up to at least 6 (Williams & Sanger, 1959). These authors suggested (cf. also Rabinowitz & Lipmann, 1960) that sequences of this type were responsible for the ability of such phosphoproteins to act as phosphate acceptors for protein phosphokinases of mammalian origin (Burnett & Kennedy, 1954) or from yeast (Rabinowitz & Lipmann, 1960). Since preparations of cerebral phosphoprotein also accepted phosphate in a suitable phosphorylating system and yielded phosphorylserine on acid hydrolysis (Heald, 1961*a*) it was of interest to see whether sequences of phosphorylserine similar to those existing in casein and phosvitin were present. The results described below show that such sequences do in fact exist in the brain phosphoproteins.

MATERIALS AND METHODS

Tissue preparations. Slices of guinea-pig cerebral cortex were cut and incubated with radioactive phosphate as described by Heald (1959). After incubation the slices were removed and dispersions made and centrifuged to yield a 'nuclear fraction' and a 'supernatant' under the conditions described in that paper. After separation the fractions were precipitated by the addition of trichloroacetic acid (10%, w/v) and extracted to yield a residue containing phosphoprotein as described previously (Heald, 1957).

Preparation of partial acid hydrolysate. Phosphoproteins were partially hydrolysed by incubation for 18 hr. at 37° with conc. HCl. The HCl was removed under vacuum over P_2O_5 and KOH and the residues were dissolved in small volumes of water. With partial hydrolysates of phosvitin these solutions could be used directly for electrophoresis. With partial hydrolysates of other proteins the hydrolysates were first treated as described by Flavin (1954). Thus the aqueous solutions, usually purple in colour, were run on to a column (2 cm.^{2} × 12 cm.) of Dowex 50 (H^+ form; 80–100 mesh) and eluted with water until the purple band had been removed. This also removed some phosphorylated peptides but the majority remained on the column and were removed with 150 ml. of 0·05 N-HCl. The eluates were reduced to small volume in a rotary evaporator at 35° and the final traces of water and HCl were removed under vacuum and over P_2O_5 and KOH. Peptides containing phosphorylserine were further partially hydrolysed to yield phosphorylserine by heating at 100° for 6 hr. in 2 N-HCl. Total hydrolysis to yield amino acids was accomplished by heating at 100° for 24 hr. in 6 N-HCl.

Electrophoresis. Samples were subjected to electrophoresis on Whatman no. 20 or 3 MM paper with formic acid–water (1:4, v/v), adjusted to pH 1·5 with NaOH, as the buffer system (Williams & Sanger, 1959). Papers (50 cm. long) were placed in horizontal electrophoresis tanks and a potential of 8–10 v/cm. was applied for 16–18 hr. at 2°. The papers were then removed and dried at 60°. Phosphorylated peptides were located with ninhydrin (0·1%, w/v) in acetone, and with the ferric chloride–sulphosalicylic acid reagent of Wade & Morgan (1954) for the detection of phosphates. To prepare sufficient quantities of peptides for further examination, suitable guide strips were used to indicate the position of the peptides on the bulk of the paper, and the corresponding areas were cut out and eluted by capillary elution with water.

Chromatography. Amino acids were separated on Whatman no. 1 paper with phenol–water (60:40, w/v) and butan-1-ol–acetic acid–water (40:10:50, by vol.) as the solvents. Phosphorylserine was separated from inorganic phosphate by chromatography on paper with the acetate–ethanol solvent of Kennedy & Smith (1954).

Phosphoproteins and albumin. Phosvitin was obtained from Nutritional Biochemicals Corp., Calif., U.S.A. All the phosphorus was liberated by alkali in 18 hr. (Rose & Heald, 1961). Pepsin, twice recrystallized, was obtained from the Sigma Chemical Co. α-Casein, prepared from Hammarsten casein (Hopkin and Williams Ltd.) was a gift from Mr S. P. R. Rose. Crystalline bovine serum albumin was obtained from Armour Laboratories Ltd. Phospholipoprotein from ox brain was prepared as described by Heald (1961*a*), 288 g. of brain being used for each preparation. The content of alkali-labile phosphate was

* Part 2: Heald (1961*a*).

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0·05–0·06% of the dry wt. for each preparation. Before partial hydrolysis lipids were removed by extraction with ether–ethanol (1:3, v/v) for 2–3 hr. at room temperature and the lipid-free residue was air-dried.

Radioactive phosphate. This was obtained as carrier-free radioactive phosphate from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Inorganic and total phosphorus. These were determined by methods described by Heald (1959).

Serine. This was determined in the total hydrolysates of the serine peptides by the method of Troll & Cannan (1953). Loss of serine during hydrolysis was corrected for by including standard serine solutions in the procedure.

Determination of radioactivity. Samples in solution (10 ml.) were counted with a M6H liquid counter (20th-Century Electronics Ltd.). Radioactivity on paper chromatograms was determined either by cutting out areas and counting under an end-window counter or by means of a gas-flow counter fitted to an automatic recording unit.

RESULTS

Although the buffer system described by Williams & Sanger (1959) ensured that only phosphorylserine, inorganic phosphate, pyrophosphate or peptides containing two or more phosphate groups migrated towards the anode, the separations obtained by these authors made use of high-voltage electrophoresis. Since this type of apparatus was not available the possibility of similar separations being obtained at low voltage was examined.

When a partial hydrolysate of phosvitin was subjected to electrophoresis for 6–7 hr. under the conditions described a series of 'bands' were detected which reacted with both peptide and phosphate reagents. Better separation was obtained if the electrophoresis was carried out for 16–18 hr. at 2°. The major ninhydrin-positive bands corresponded exactly with those containing phosphate (Fig. 1). In all, six bands were detected. Phosphorylserine migrated at the same rate as band 2, and inorganic phosphate migrated to a position slightly in front of band 6, causing some contamination. Pyrophosphate migrated ahead of inorganic phosphate. When bands 1–5 were separately eluted and again subjected to electrophoresis only one component, migrating at the same rate as the parent band, was obtained from each fraction. The bands were partially hydrolysed by conc. HCl and the partial hydrolysates were again separated by electrophoresis at pH 1·5. Bands 3–5 yielded fractions migrating at the same rate as the phosphorylserine in addition to the intermediate bands (Fig. 2). Band 1 was partially stable but also yielded phosphorylserine and a trace of band 3, whereas band 2 was largely stable but yielded a trace of band 1. Chromatography of the total acid hydrolysates showed that serine was the only major detectable amino acid present in each fraction together with possible traces of glutamic acid. The major bands therefore represent phosphoserine peptides of the type described by Williams & Sanger (1959) probably intermixed, particularly in bands 1 and 2, with phosphorylserine peptides of a higher molecular weight but partially dephosphorylated as a result of the acid hydrolysis. The presence of such dephosphorylated peptides is supported by estimations of the serine/phosphorus ratio in each fraction. These were: band 1, 2·15; band 2, 1·72; band 3, 1·22; band 4, 1·27; band 5, 1·19. The ratio of serine to phosphate exceeded unity in all fractions and tended to decrease as the complexity of the phosphopeptide increased. This trend would be expected since the peptides represented by band 5 are more likely to be closer to the size of the actual sequence in the phosphoprotein than are the peptides in band 1. Since the bands contained only serine and phos-

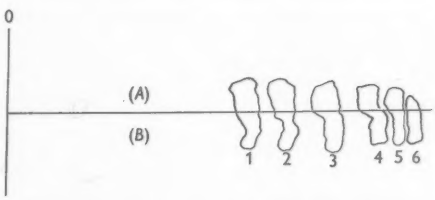

Fig. 1. Separation of phosphopeptides from acid hydrolysates of phosvitin by electrophoresis in formic acid (20%, v/v)–NaOH, pH 1·5. Conditions were as described in the Materials and Methods section. (*A*) Sprayed with ninhydrin; (*B*) sprayed with the phosphate reagents of Wade & Morgan (1954). This and the following Figures are copies of tracings taken from the original electrophoretograms.

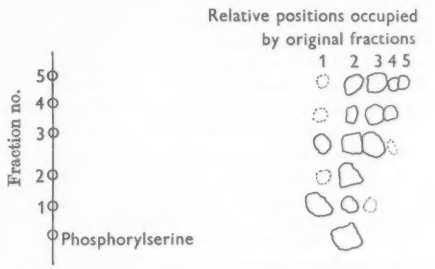

Fig. 2. Electrophoresis of fractions 1–5 (Fig. 1) after further partial acid hydrolysis. System was as described in Fig. 1. Electrophoresis was for 6 hr. at 10 v/cm. The fractions were detected with ninhydrin. Broken lines indicate the position of areas of weak reaction.

phorus it is considered that a serine/phosphorus ratio greater than unity is indicative of the presence of polyserylpeptides in which only some of the seryl residues are phosphorylated. The degree of polymerization could not be assessed, but, by analogy with the conclusions of Williams & Sanger (1959), bands 3, 4, 5 and 6 must represent a series in which $(Ser\text{-}P)_2$ is the minimum. It is not known whether the peptides described by these authors contained equivalent amounts of phosphorus and serine.

Partial hydrolysis of α-casein and of unfractionated casein also yielded phosphopeptides migrating at a rate similar to those from phosvitin (Fig. 3). In most instances bands 5 and 6 were not visible. This was not due to the smaller amounts of phosphorylated fragments derived from casein, containing 0·8–0·9% of P, as compared with phosvitin containing 8–10% of P, since partial hydrolysis of 0·5 g. of casein and a separation of the total phosphopeptides in the hydrolysate failed to reveal band 6, though band 5 was then visible. On elution and total hydrolysis, serine was again the major amino acid detected, accompanied by smaller amounts of alanine, leucine and glutamic acid. Similar amino acids were found by Williams & Sanger (1959) in fractions from casein migrating at rates intermediate to the main phosphoseryl peptides.

The above experiments showed that low-voltage electrophoresis, though not achieving the degree of resolution of the high-voltage system, was nevertheless capable of separating groups of peptides containing phosphorylserine polymers, from partial hydrolysates of casein and phosvitin. It was therefore used to examine brain phosphoprotein.

Partial hydrolysis of brain phosphoproteins. Lipid-free preparations of brain phosphoprotein were partially hydrolysed and the phosphopeptides separated on Dowex 50 and subjected to electrophoresis. The fractions obtained migrated at rates similar to those obtained from casein and phosvitin (Fig. 3). Four bands were visible. However, the quantities of phosphoprotein phosphorus were extremely small and separations of the type shown in Fig. 3 necessitated placing all the phosphopeptides derived from a fraction (representing 100–150 g. of fresh brain) on to the paper. To reduce laborious preparations the technique of isotope dilution was used. Phosphoserine from brain phosphoproteins is radioactive when derived from tissues incubated with ^{32}P (Heald, 1958). Slices of guinea-pig cerebral cortex were incubated with radioactive phosphate and the phosphoprotein fractions obtained and partially hydrolysed in the presence of phosvitin (about 20 mg. of phosvitin for 700 mg. of slices). The partial hydrolysates were then subjected to electrophoresis at pH 1·5. When the paper strips were scanned in an automatic scanner the areas of radioactivity corresponded closely with the major ninhydrin-reacting bands present (Fig. 4). To determine whether the radioactivity represented phosphoseryl peptides, the areas corresponding to the bands were eluted and partially hydrolysed in 2N-HCl at 100° in the presence of carrier phosphorylserine. The hydrolysates were then separated both by electrophoresis and by chromatography on paper. The areas containing phosphorylserine were removed and counted; they contained up to 30% of the radioactivity added to the paper (Table 1). The remaining activity was concentrated largely in the areas corresponding to inorganic phosphate. Inclusion of radioactivity in phosphorylserine was not due to chemical exchange since an experiment in which phosvitin was partially hydrolysed with 40 μc of inorganic $[^{32}P]$phosphate revealed radioactivity, after separation of the phosphopeptides, only in the areas of ortho- and pyro-phosphate.

Fig. 3. Comparisons of the rates of migration of phosphopeptides derived from brain phosphoprotein with phosphopeptides derived from α-casein and phosvitin. Separation and conditions were as described in Fig. 1.

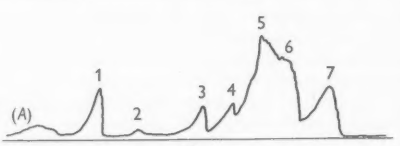

Fig. 4. Radioactivity in phosphopeptides separated from partial acid hydrolysates of radioactive brain phosphoprotein, admixed with phosvitin before partial hydrolysis. Conditions were as described in Fig. 1. (*A*) Tracing of the curve of radioactivity drawn by an automatic recording apparatus; (*B*) position of the phosphopeptides detected with ninhydrin. Peaks 6 and 7 correspond to the position of orthophosphate and pyrophosphate respectively. For this recording peak 7 was recorded with the apparatus set for a full-scale deflexion with 1000 counts/sec. and the remainder with a setting for a full-scale deflexion with 300 counts/sec. The highest point of peak 5 corresponds approximately to an activity of 200 counts/sec.

Table 1. *Radioactivity of phosphorylserine derived from phosphopeptides in partial acid hydrolysates of brain phosphoprotein*

Slices of guinea-pig cerebral cortex were incubated with radioactive phosphate and the phosphoprotein fraction (see Materials and Methods section) was partially hydrolysed together with phosvitin. The partial hydrolysate, after removal of HCl, was subjected to electrophoresis at pH 1·5 and the phosphopeptides so obtained were eluted and hydrolysed with 2N-HCl for 6 hr. at 100° in the presence of carrier phosphorylserine. The hydrolysates were subjected to electrophoresis at pH 1·5 and the areas corresponding to phosphorylserine cut out and counted. Fraction numbers are as given in Fig. 1.

Source of tissue phosphoprotein	Fraction no.	Counts/min. added to paper	Counts/min. in phosphoryl-serine	Activity in phosphoryl-serine as % of activity added
'Nuclear' fraction	1	1236	383	31·0
	2	2415	611	25·0
	3	660	176	26·6
	4	452	137	30·3
	5*	1110	212	19·3
	6*	4200	103	2·5
'Supernatant' fraction	1	1000	247	24·7
	2	3040	775	25·5
	3	339	228	67·0†
	4	1540	444	28·6

* Includes inorganic phosphate contamination of original fractions.
† High percentage count is anomalous.

DISCUSSION

The results presented above establish the existence, in brain phosphoproteins, of sequences of phosphorylserine residues of a type similar to those found in casein and phosvitin. This conclusion is based on the following: (*a*) partial acid hydrolysis of brain phosphoprotein yielded phosphopeptides migrating at rates similar to those from phosvitin and casein; (*b*) when brain phosphoprotein was made radioactive by incorporation of radioactive phosphate and partially hydrolysed in the presence of phosvitin the phosphoseryl peptides isolated contained radioactive phosphorus; (*c*) when these peptides were hydrolysed to yield phosphorylserine, the phosphorylserine separated both chromatographically and electrophoretically was radioactive. In the absence of isolation and analysis of the individual peptides the presence or absence of other amino acids cannot be decided upon conclusively, but the similarities of migration on electrophoresis at pH 1·5 of the peptides from brain with those from phosvitin and casein suggest that other amino acids formed only a small fraction of the total peptide.

Previous work (Heald, 1959) has shown that when the intact tissue is electrically stimulated only the phosphoprotein appearing in the 'nuclear' fraction increased in radioactivity, whereas that present in the 'supernatant' was not affected. Since both the 'nuclear' and the 'supernatant' fraction (the latter containing mitochondria) contained phosphoproteins yielding phosphoseryl peptides upon hydrolysis, it is considered that the distinction in metabolism resulting from the electrical stimulus is more probably a reflexion of function than of type. The function of cellular phosphoproteins of a non-enzymic type is not known, though suggestions of involvement in ion transport (Heald, 1960, 1961*b*) or water movement (Judah, 1960) have been made. In these connexions it seems highly significant that the presence of sequences of negatively charged phosphate groups places the phosphoproteins of brain in the category of natural polyelectrolytes which in solution are capable of acting as do ion-exchange resins. Since brain phosphoproteins are capable of being both phosphorylated and dephosphorylated (Heald, 1961*a*; Rose & Heald, 1961) by cerebral enzymes, it is suggested that in the cell they operate as metabolically activated natural ion-exchange materials, the results of the activation being the transport of ions or water across cellular membranes. The implications of this type of system are being investigated.

SUMMARY

1. By use of low-voltage paper electrophoresis of partial acid hydrolysates of phosphoproteins, it has been confirmed that casein and phosvitin contain sequences of phosphorylseryl residues of the type $(\text{Ser-P})_n$.

2. The fractions separated by electrophoresis, though migrating as single components, were not pure and contained phosphoseryl peptides linked with other amino acids, probably glutamic acid and partially dephosphorylated phosphoseryl peptides.

3. Phosphoproteins of brain contain similar phosphoseryl sequences. This has been shown both by comparison of rates of migration of phosphopeptides separated from partial hydrolysates and by a technique involving isotopic dilution.

4. The results are discussed in relation to the possible metabolic role of brain phosphoproteins in the intact tissue.

I am indebted to Dr J. Williams for helpful correspondence during the initial stages of this work, and Dr M. J. H. Smith, Kings College Hospital Medical School, for permission to use an automatic recording apparatus (constructed on the premises) for the detection of radioactivity in chromatographic strips.

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The Action of a *Lactobacillus bifidus* Dextranase on a Branched Dextran

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It has been shown that an extracellular dextranase of a rumen strain of *Lactobacillus bifidus*, when incubated with the essentially unbranched dextran of *Streptococcus bovis*, produces isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose and traces of isomaltoheptaose, but no glucose or isomaltose (Bailey & Clarke, 1959). A study of the action of the *L. bifidus* dextranase on various dextrans revealed that the mixture of oligosaccharides produced varied with the type, and degree of branching, of the dextran (Bailey, Hutson & Weigel, 1960). We have now examined in detail the action of this dextranase on the branched dextran elaborated by *Leuconostoc mesenteroides* (*Betacoccus arabinosaceous*, Birmingham strain).

EXPERIMENTAL

Dextranase. The dextranase was isolated from cell-free culture fluid of a rumen strain of *Lb. bifidus* by one of us (R.W.B.) in the Laboratories of the Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand, according to the method described by Bailey & Clarke (1959).

Dextrans. Leuconostoc mesenteroides (Birmingham strain) dextran was synthesized from sucrose. The dextran was from the same batch as was that used for structural studies of the dextran, which was shown to contain, as well as α-1:6-linkages, 12–15% of α-1:3-branch linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954). A virtually unbranched dextran (Bailey, 1959) was isolated from a sucrose-containing culture of *S. bovis* (strain I) (Bailey & Oxford, 1958).

Paper chromatography and ionophoresis. The solvents used for paper chromatography were: (*a*) the upper layer of ethyl acetate–water–pyridine (2:2:1, by vol.) (Jermyn & Isherwood, 1949); (*b*) ethyl acetate–water–pyridine–acetone (Malpress & Hytten, 1958); (*c*) upper layer of butanol–ethanol–water (4:1:5, by vol.); (*d*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*e*) butanol–benzene–pyridine–water (5:1:3:2, by vol.). Ionophoresis was carried out at about 50 v/cm. in borate solution, pH 10 (Foster, 1953), and in molybdate solution, pH 5·5 (Bourne, Hutson & Weigel, 1959). It will be noticed that some of the M_S (mobility with respect to sorbitol) values reported here differ slightly from those reported earlier. This is due to the application of smaller quantities, thus allowing a more accurate determination of the rates of migration. In all cases comparison was made with known compounds.

The reagents used for the detection of compounds were: (*a*) silver nitrate in acetone–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950); (*b*) aniline hydrogen phthalate (Partridge, 1949); (*c*) *p*-anisidine–HCl (Hough, Jones & Wadman, 1950); (*d*) aniline–diphenylamine–

phosphoric acid (Schwimmer & Bevenue, 1956); (*c*) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956).

Dextranase digests. Standard digests were prepared from dextran solution (15 mg. in 2 ml. of water), dextranase solution (5 mg. in 1 ml. of water) and 0·2 M-citrate buffer, pH 5·5 (2 ml.). The digests were incubated under a layer of toluene at 37° for 60 hr.

Reducing sugars. Reducing sugars produced were determined at time intervals by the cuprimetric method of Shaffer & Hartmann (1921). The results were calculated in terms of isomaltotriose (Fig. 1). The solutions were desalted with Bio-Deminrolit, pretreated with carbon dioxide and evaporated to dryness *in vacuo*. The residues were dissolved in water (0·2 ml.) for chromatographic analysis.

RESULTS

Oligosaccharides produced by the action of the dextranase on Leuconostoc mesenteroides *dextran*

The rate of liberation of reducing sugars from *L. mesenteroides* dextran by *Lb. bifidus* dextranase is compared with that from *S. bovis* dextran in Fig. 1. The amounts of reducing sugars released indicates the effect of branching on the enzymic hydrolysis. Paper chromatography in solvent (*b*) of the digest containing *L. mesenteroides* dextran revealed, after 30 hr. incubation, the formation of three oligosaccharides, A, B and C, with $R_{\text{isomaltotriose}}$ (R_{IMT}) values of 0·75, 0·50 and 0·35 respectively, in addition to isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose and isomaltoheptaose, also obtained from *S. bovis* dextran. Glucose, isomaltose and oligosaccharides with a degree of polymerization greater than seven glucose units were absent from the digest containing *S. bovis* dextran. Glucose and isomaltose were also absent from the digest containing *L. mesenteroides* dextran, but a large amount of unresolvable material with $R_{\text{IMT}} < 0{\cdot}20$ was produced.

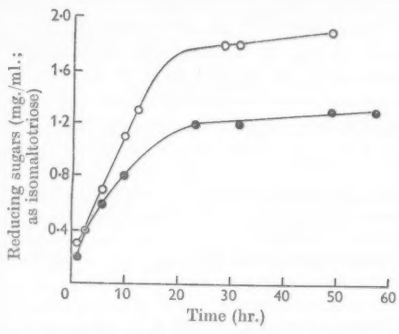

Fig 1. Liberation of reducing sugars in dextran–dextranase digests. Solutions contained dextran (7·5 mg./ml.; 10 ml.), dextranase (5 mg./ml.; 5 ml.) and citrate buffer (0·2 M, pH 5·5; 10 ml.). ○, *S. bovis* dextran; ●, *L. mesenteroides* dextran.

Table 1. *Yields of oligosaccharides produced by the action of* Lb. bifidus *dextranase on* Leuconostoc mesenteroides (*Birmingham strain*) *dextran*

Solutions containing dextran (15 mg./2 ml. of water), dextranase (5 mg./1 ml. of water) and citrate buffer (0·2 M, pH 5·5; 2 ml.) were incubated at 37° for 60 hr. Oligosaccharides were resolved by chromatography in solvent (*b*) and determined by the anthrone method. Grading scheme in visual estimation: + (trace) to ++++ (intense).

	L. mesenteroides dextran (% of recovered material)	*S. bovis* dextran (by visual estimation)
Isomaltotriose	8·8	++++
Isomaltotetraose	9·4	++++
Tetrasaccharide A	3·3	Absent
Isomaltopentaose	11·4	++++
Pentasaccharide B	3·5	Absent
Hexasaccharide fraction	8·6	+++
Heptasaccharide fraction	13·1	+
Unresolvable fraction	41·9	Absent

A part of the digest containing *L. mesenteroides* dextran (3·76 mg. of the original dextran; corrected for moisture content) was fractionated by paper chromatography in solvent (*b*). The glucose equivalent of each eluted fraction was determined by the anthrone method (Yemm & Willis, 1954). The results are shown in Table 1. Qualitative results obtained from *S. bovis* dextran are included for comparison.

A digest (500 ml.) containing *L. mesenteroides* dextran (1·90 g.) was incubated under standard conditions. After desalting, the mixture of oligosaccharides was fractionated by paper chromatography in solvent (*b*) on Whatman no. 3 filter paper. Purification of the fractions containing oligosaccharides A and B by the same paper-chromatographic method, followed by desalting and freeze-drying, yielded chromatographically pure oligosaccharide A (30 mg.) and B (30 mg.).

Structural examination of oligosaccharide A. Oligosaccharide A could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*) (green spot) and (*d*) (pink spot). During ionophoresis in borate solution (pH 10) it migrated with the same M_G (mobility with respect to glucose) as isomaltotetraose (0·65). When estimated by the Shaffer & Hartmann (1921) method oligosaccharide A (5 mg.) had a reducing power equivalent to 1·15 mg. of glucose. This corresponded to 85·2 % of the calculated value for a tetrasaccharide. It was not possible to determine the ash and moisture contents.

Oligosaccharide A (250 μg.) was heated for 4 hr. at 100° in 1·5 N-HCl (1 ml.) and the solution evaporated to dryness *in vacuo* over solid NaOH to

remove the acid. The hydrolysate contained a single component which was chromatographically identical with glucose. A partial hydrolysate, obtained by heating for 1 hr. at 100° in N-HCl and deionizing with Amberlite IRA-400 (carbonate), was shown by paper chromatography to contain isomaltotriose, isomaltose and glucose.

Oligosaccharide A (12 mg.) was reduced with potassium borohydride (Bragg & Hough, 1957). The solution was deionized by treatment with Amberlite IR-120 (H^+) followed by evaporation and repeated distillation with dry methanol. Oligosaccharide A alcohol was purified and isolated by paper chromatography in solvent (*a*) (R_G 0·32). Ionophoresis in molybdate solution (pH 5·5) (Bourne *et al.* 1959) showed that it migrated with the same M_S as 6-*O*-α-isomaltotriosylsorbitol (0·50).

Oligosaccharide A alcohol was partially hydrolysed by heating for 4 hr. at 100° in 1% aqueous oxalic acid (5 ml.). The hydrolysate was deionized with Amberlite IRA-400 (carbonate) and fractionated by paper chromatography in solvent (*a*) (see Table 2) into 5 components in addition to unchanged material (about 40%, visual estimation on chromatograms). Each fraction was subjected to ionophoresis in molybdate solution (pH 5·5), when fractions 3, 4 and 6 were resolved into trisaccharide D and trisaccharide E alcohol, isomaltose and 6-*O*-α-D-glucopyranosylsorbitol, D-glucose and sorbitol respectively. The identity of the products of hydrolysis with the corresponding named compounds was confirmed by ionophoresis in borate solution.

Trisaccharide D, which was immobile during ionophoresis in molybdate solution (pH 5·5), was eluted and, after removal of molybdate with Amberlite IR-120 (H^+) and Amberlite IRA-400 (carbonate), reduced with potassium borohydride (Bragg & Hough, 1957). The solution was deionized as described above. Ionophoresis in molybdate solution (pH 5·5) revealed the presence of trisaccharide F alcohol (M_S 0) and trisaccharide E alcohol (M_S 0·62).

To separate solutions of oligosaccharide A (500 μg.) and isomaltotetraose (500 μg.) in water (1 ml.) was added 0·03 M-sodium periodate (0·5 ml.). After standing for 16 hr. at room temperature the excess of periodate was destroyed with 0·03 M-ethylene glycol (0·5 ml.). Hydrochloric acid was added to give 2 N-HCl solutions. These were heated for 6 hr. at 100° and then evaporated *in vacuo* over solid NaOH. Although paper chromatography in solvent (*a*) of the hydrolysate of oxidized oligosaccharide A revealed an intense spot of glucose, the hydrolysate of oxidized isomaltotetraose was shown to contain only traces of glucose. Measured portions of the hydrolysates (containing 250 μg. of original oligosaccharides) were fractionated by paper chromatography in solvent (*a*). The components corresponding to glucose were eluted with water and determined by the benzidine method (Jones & Pridham, 1954), with glucose as a standard. The yields of glucose from oligosaccharide A and isomaltotetraose corresponded to 27% and 5% respectively of the glucose present in a tetrasaccharide containing glucose only.

Structural examination of oligosaccharide B. The methods were as for oligosaccharide A. Oligosaccharide B could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*) (green spot) and (*d*) (pink spot). During ionophoresis in borate solution (pH 10) it migrated with the same M_G as isomaltopentaose. Complete hydrolysis gave a single reducing compound which was chromatographically identical with glucose, whereas a partial hydrolysate was shown to contain, by paper chromatography in solvents (*a*) and (*b*), isomaltotetraose, isomaltotriose, isomaltose and glucose. Oligosaccharide B alcohol migrated during ionophoresis in molybdate solution (pH 5·5) with the same M_S as 6-*O*-α-isomaltotetraosylsorbitol (0·44). A partial acid hydrolysate of oligosaccharide B alcohol showed, on chromato-

Table 2. *Fractionation of products from partial hydrolysis of oligosaccharide A alcohol*

Paper chromatography in solvent (*a*)		Ionophoresis in molybdate solution	
Fraction no.	R_G	M_S	Identity
1	0·32	0·50	Oligosaccharide A alcohol
2	0·45	0·60	6-*O*-α-Isomaltosylsorbitol
3	0·55	0	Trisaccharide D
		0·60	Trisaccharide E alcohol
4	0·75	0	Isomaltose
		0·75	6-*O*-α-D-Glucopyranosylsorbitol
5	0·85	0	Nigerose
6	1·0	0	Glucose
		1·0	Sorbitol

graphy in solvents (*a*) and (*d*), in addition to unchanged material (about 50%, visual estimation), intense spots corresponding to glucose and 6-*O*-α-isomaltotriosylsorbitol. Only very small traces of isomaltose, 6-*O*-α-D-glucopyranosylsorbitol and sorbitol were detectable.

When oligosaccharide B (500 μg.) was oxidized with sodium periodate and hydrolysed in the same way as oligosaccharide A and isomaltotetraose, the yield of glucose corresponded to 24% of the glucose present in a pentasaccharide containing only glucose.

Action of the dextranase on 6-O-α-*isomaltohexaosyl- and* 6-O-α-*isomaltoheptaosyl-sorbitol*

6-*O*-α-Isomaltohexaosylsorbitol (8 mg.) and 6-*O*-α-isomaltoheptaosylsorbitol (8 mg.), prepared by reduction of isomaltoheptaose and isomaltooctaose respectively with potassium borohydride, were separately dissolved in 0·25M-citrate buffer (pH 5·5) (1 ml.) and incubated with dextranase (4 and 6 mg. respectively) for 26 hr. at 37°. Paper ionophoresis in molybdate solution of the deionized digest containing 6-*O*-α-isomaltohexaosylsorbitol revealed, in addition to non-migrating material, the presence of two components which migrated at the same rate as 6-*O*-α-isomaltosyl- and 6-*O*-α-isomaltotriosyl-sorbitol respectively. The non-migrating material was eluted from the paper and deionized. Paper chromatography in solvent (*a*) revealed components corresponding to isomaltotriose and isomaltotetraose. 6-*O*-α-Isomaltosylsorbitol, 6-*O*-α-isomaltotriosylsorbitol, 6-*O*-α-isomaltotetraosylsorbitol, isomaltotriose, isomaltotetraose and isomaltopentaose were identified in a similar manner in the digest containing 6-*O*-α-isomaltoheptaosylsorbitol. Visual examination of the paper ionograms and chromatograms suggested that all the products were present in approximately equal quantities.

DISCUSSION

The evidence presented shows that oligosaccharide A is a reducing tetrasaccharide of glucose. The results of the partial acid hydrolysis of tetrasaccharide A and the reduction product showed the presence of an isomaltotriose unit, together with another glucose unit joined by a linkage of a more labile type. One glucose unit was shown to be resistant to oxidation by periodate. This demonstrates the presence of one 1:3-glucosidic linkage in tetrasaccharide A. As the 1:3- as well as the 1:6-glucosidic linkages in dextran are of the α-type it is reasonable to assume that the 1:3-glucosidic linkage in tetrasaccharide A also is an α-1:3-linkage.

There are four possible structures (I–IV) (Fig. 2) for a tetrasaccharide containing an isomaltotriose unit to which a fourth glucose unit is joined by an α-1:3-glucosidic linkage. The results of periodate oxidation eliminate structures (III) and (IV). The migration of tetrasaccharide A alcohol during ionophoresis in molybdate solution conclusively eliminates structures (III) and (IV) because a 3-*O*-substituted sorbitol does not form a complex with molybdate (Bourne *et al.* 1959). This migration and the reaction of tetrasaccharide A with triphenyltetrazolium chloride also eliminate the possibility of substitution at $C_{(4)}$- and $C_{(2)}$-hydroxyl groups respectively of the reducing glucose unit.

The structure of tetrasaccharide A was elucidated further by a partial hydrolysis of the reduction product (V or VI) (Fig. 3). Products which would be expected from both structures, namely 6-*O*-isomaltosylsorbitol (VII), isomaltose (XII), 6-*O*-α-D-glucopyranosylsorbitol (XIII), nigerose (XIV), glucose (XV) and sorbitol (XVI), were identified by paper chromatography and ionophoresis. As would be expected if (VI) were present, two trisaccharide alcohols were obtained (Table 2). One of these (fraction 2) was identical with 6-*O*-isomaltosylsorbitol (VII), the only trisaccharide alcohol expected from (V). The other, trisaccharide E alcohol, must have structure (IX), which is consistent with its M_S.

The possible structures of the reducing trisaccharide D of fraction 3 are (VIII) and (X). Ionophoresis in molybdate solution (pH 5·5) of the reduced trisaccharide D revealed trisaccharide E alcohol (IX) and a non-migrating trisaccharide F alcohol. As 3-*O*-substituted sorbitols do not migrate, trisaccharide F alcohol must have structure (XI), and hence trisaccharide D must have contained both (VIII) and (X).

These results show that tetrasaccharide A is a mixture of 3^3-glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II).

The results obtained with oligosaccharide B provide evidence that it is a reducing pentasaccharide B containing an isomaltotetraose unit to which a fifth glucose unit is joined by a 1:3-

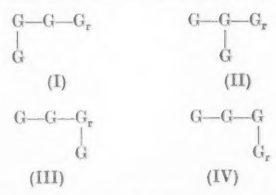

Fig. 2. Possible structures of a tetrasaccharide containing an isomaltotriose unit and a fourth glucosyl unit joined by an α-1:3-link. —, α-1:6-link; |, α-1:3-link; G , reducing glucose unit; G, glucosyl unit.

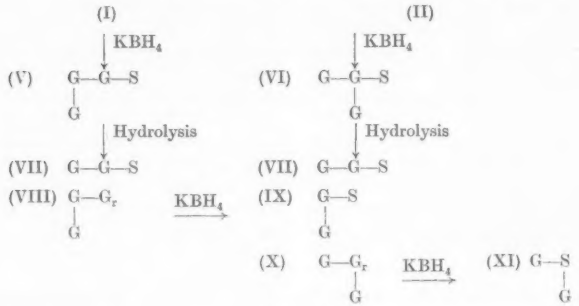

Fig. 3. Products of reduction-hydrolysis of tetrasaccharides (I) and (II). —, α-1:6-link; |, α-1:3-link, G_r, reducing glucose unit; G, glucosyl unit; S, sorbitol.

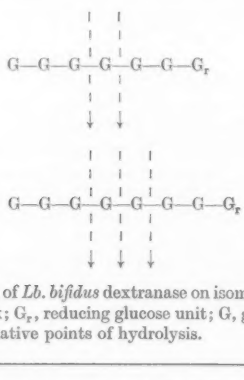

Fig. 4. Action of *Lb. bifidus* dextranase on isomaltodextrins, —, α-1:6-Link; G_r, reducing glucose unit; G, glucosyl unit; – – →, alternative points of hydrolysis.

glucosidic linkage similar to that in (I) or (II). The fact that tetrasaccharide A is a mixture of 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose suggests that pentasaccharide B is also a mixture of pentasaccharides with these types of structures.

Tetrasaccharides (I) and (II) and pentasaccharide B could have arisen by enzymic hydrolysis of *L. mesenteroides* dextran or by chemical or enzymic transglucosylation action on isomaltodextrins. The latter possibility is unlikely as, in this case, they would be expected to have been produced in the control digest containing *S. bovis* dextran. The presence of a 1:3-glucosidic linkage in tetrasaccharides (I) and (II) and pentasaccharide B indicates that the enzyme cannot hydrolyse the 1:3-linkage. We believe that the present work is the first occasion in which oligosaccharides containing the branch linkages of the original dextran have been found.

Bailey & Clarke (1959) established that the action of *Lb. bifidus* dextranase involves random hydrolysis of the dextran chain and that the smallest isomaltodextrin readily hydrolysed is isomaltoheptaose. The action of the enzyme on 6-*O*-α-isomaltohexaosyl- and 6-*O*-α-isomaltoheptaosylsorbitol shows that it has no preference for a particular type of chain end, a result which is in accordance with the properties of an endopolysaccharase. It is probable, therefore, that the dextranase hydrolyses dextran or an isomaltodextrin containing not less than seven glucose units at a point not less than three glucosidic linkages from a chain end, as shown in Fig. 4.

The production of 3^3-glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II) and pentasaccharide B suggests that this type of hydrolysis of dextran by the dextranase is not inhibited by the presence of 1:3-branch linkages. The structure of these products and the absence from the digest of oligosaccharides in which the reducing glucose unit is substituted at $C_{(3)}$ suggests that the α-1:6-link on the reducing side of the branch point of the main dextran chain is resistant to hydrolysis by the dextranase. The branching of *L. mesenteroides* dextran has been reported to occur once in each 6 or 7 anhydroglucose units (Barker *et al.* 1954). It can be calculated that dextrans of regular structures

with branch links on every sixth, seventh and eighth anhydroglucose unit respectively will yield, on hydrolysis with *Lb. bifidus* dextranase, oligosaccharides in unequal proportions. The approximately equal quantities of isomalto-triose, -tetraose and -pentaose produced indicate that the branching of this dextran is random. The presence in the digest of much material containing more than six glucosyl units is further support for a random branching of the dextran molecule.

It is significant that the 'branching' in tetrasaccharide A and pentasaccharide B consists of only one glucosyl unit. 3^3-Glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II) could arise from a part of the dextran molecule where the branching was a single glucosyl unit, or from parts of a randomly branched dextran molecule. Bovey (1959), on the grounds of physical measurements, has suggested that 80% of the branches in *L. mesenteroides* (NRRL-B512) dextran consist of only one glucosyl unit.

SUMMARY

1. *Lactobacillus bifidus* dextranase has been shown to hydrolyse *Leuconostoc mesenteroides* (Birmingham strain) dextran to a complex mixture of oligosaccharides.

2. In addition to isomalto-triose, -tetraose, -pentaose, -hexaose and unresolvable material of degree of polymerization greater than 6, a tetrasaccharide (A) and a pentasaccharide (B) were isolated.

3. Tetrasaccharide (A) was shown to be a mixture of 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose. Pentasaccharide (B) contained one glucose unit joined through a 1:3-linkage to a glucose unit, other than the reducing one, of isomaltotetraose and is probably a mixture of isomers.

4. The dextranase has been shown to hydrolyse 6-*O*-α-isomaltohexaosyl- and 6-*O*-α-isomaltoheptaosyl-sorbitol at two and three alternative glucosidic linkages respectively.

5. The implications of the results, as far as dextranase action and dextran structure are concerned, have been discussed.

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The Purification and Properties of a Factor containing Vitamin B_{12} concerned in the Synthesis of Methionine by *Escherichia coli*

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Evidence has been obtained that cobalamin and folic acid are concerned in the synthesis of methionine from homocysteine by *Escherichia coli* strain PA15, a serine- or glycine-requiring auxotroph (Gibson & Woods, 1960; Cross & Woods, 1954; Szulmajster & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). (In this paper, the terms folic acid and vitamin B_{12} will be used in general reference to the two families of compounds; names in common use will be used in reference to specific

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members of these families, e.g. tetrahydropteroylglutamic acid, cyanocobalamin.) The folic acid cofactor for the transfer of a C_1 unit from serine to homocysteine is supplied to cell-free enzyme-containing extracts by an extract of heated organisms. The behaviour of this heated extract is imitated by tetrahydropteroyltriglutamic acid and also by its N^5- and N^{10}-formyl derivatives; tetrahydropteroylglutamic acid is inactive and inhibits the action of the heated extract and of the synthetic tetrahydropteroyltriglutamic acids (Kisliuk & Woods, 1960; Guest *et al.* 1960; Jones, Guest & Woods, 1961). However, tetrahydropteroylglutamic acid is used as the folic acid cofactor by extracts of acetone-dried organisms if these organisms are harvested from a growth medium containing cobalamin (Kisliuk & Woods, 1957, 1960). Ultrasonic extracts of strain PA15 use tetrahydropteroylglutamic acid as cofactor if free cobalamin is added to the reaction mixture; there is no requirement for cobalamin with the extract of heated organisms as a source of folic acid (Guest *et al.* 1960). However, ultrasonic extracts of *E. coli* strain 121/176 (a cobalamin- or methionine-requiring auxotroph) require cobalamin for the synthesis of methionine when the source of folic acid is either the extract of heated *E. coli* or tetrahydropteroylglutamate (Helleiner, Kisliuk & Woods, 1957; Guest *et al.* 1960).

The presence of a non-diffusible, heat-labile material was demonstrated in extracts of acetone-dried powders of strain PA15 cultured in the presence of cobalamin (Kisliuk & Woods, 1960). Evidence has also been presented that ultrasonic extracts of *E. coli* are able to form this factor from free cobalamin, or, more efficiently, from 5:6-dimethylbenzimidazolylcobamide coenzyme (Woods, 1958; Guest, 1960; Guest & Woods, 1960). The factor, derived either from the extract of acetone-dried organisms grown in the presence of cobalamin or by incubation of free cobalamin with ultrasonic extracts, enabled tetrahydropteroylglutamic acid to be used as the source of cofactor for C_1-transfer in methionine synthesis by extracts of acetone-dried powders of the same organism grown in the absence of cobalamin; under these conditions, free cobalamin was without effect (Woods, 1958; Kisliuk & Woods, 1960; Guest & Woods, 1960). Several analogues of cobalamin inhibited the formation of the factor from cobalamin by ultrasonic extracts, but they had no effect on the activity of the factor.

In this paper a method is presented for the purification of this factor from extracts of acetone-dried organisms of strain PA15 cultured in the presence of cobalamin, together with an investigation of some of its properties.

While this work was in progress, the existence of a cobalamin-containing enzyme, forming part of the enzyme complex catalysing the synthesis of methionine from homocysteine and serine by a cobalamin- or methionine-requiring auxotroph of *E. coli*, was reported (Hatch, Takeyama & Buchanan, 1959). Brief descriptions have also appeared of the purification of this enzyme (Hatch, Cathou & Larrabee, 1960) and its formation *in vitro* from vitamin B_{12} and an apoenzyme prepared from extracts of the auxotrophic organisms grown in the absence of vitamin B_{12} (Takeyama & Buchanan, 1960). A short report has also appeared on the purification of a vitamin B_{12}–protein concerned in methionine synthesis by ultrasonic extracts of *E. coli* PA15 grown in the presence of vitamin B_{12} (Kisliuk, 1960).

MATERIALS AND METHODS

Escherichia coli strain PA15, an auxotroph requiring serine or glycine for growth, was used as a source of both the factor and the enzyme system for its assay. The organism was maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. It was originally obtained from Dr Barbara Wright and the parent strain was isolated from sewage (B. E. Wright, personal communication).

Bulk production of acetone-dried powders of organisms was carried out as described by Szulmajster & Woods (1960). Organisms cultured in the presence of cobalamin were obtained by the addition of 20 μg. of cobalamin to the inoculum for each tray containing 500 ml. of agar medium, and incubating for 18 hr. at 37°. Organisms cultured in the absence of cobalamin were incubated for 40 hr. at 37°.

The powders, in batches of about 100 g., representing the yield from 200–400 trays of medium, were stored in sealed containers at $-15°$; there was no detectable loss of activity for at least 6 months.

Preparation of extracts. Dialysed extracts of acetone-dried organisms grown in the absence of cobalamin were prepared as described by Jones *et al.* (1961). On some occasions the extracts were treated with Dowex-1 X8 resin (Cl form; 100–200 mesh) as described by Kisliuk & Woods (1960). This extract will be referred to as the assay extract.

Crude factor-containing extracts of acetone-dried organisms grown in the presence of cobalamin were prepared by mixing the powder with water (10 ml./g. of powder) in a stainless-steel Potter–Elvehjem homogenizer. The mixture was subjected for 75 sec. to ultrasonic vibration (25 kcyc./sec. at 600 w, produced by an ultrasonic generator type E7590B, Mullard Ltd., London), diluted with a further 5 ml. of water/g. of powder and then centrifuged (78 000*g*, 60 min. at 0°) in a Spinco model L ultracentrifuge. The supernatant fluid was stored at $-15°$ to 0° for up to 18 hr. before further treatment.

Estimation of protein. This was determined spectrophotometrically according to Layne (1957); the nucleic acid content was determined similarly. Measured in this way, the protein concentrations of a variety of solutions obtained during the fractionation procedure agreed, to within 10%, with values obtained by the biuret method (Layne, 1957), the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) and the method of Stickland (1951), with

crystalline bovine serum albumin (Armour Laboratories, Hampden Park, Eastbourne, Sussex) as standard.

Materials for column chromatography. Celite 545 (Johns–Manville, London) was graded to remove fine particles and washed exhaustively by decantation with large volumes of distilled water. Columns were poured as homogeneous slurries in water and washed with several column volumes of 20 mM-potassium phosphate buffer, pH 7·8.

Carboxymethylcellulose (Whatman CM-70 cellulose powder) was washed with 1 vol. of 0·1 N-HCl, 5–6 vol. of water (until free of HCl), 1 vol. of 0·5 M-KCl in 0·5 M-KOH and finally 5–6 vol. of water (until free of KOH). Columns were poured as homogeneous slurries in water and washed with 20 vol. of 160 mM-potassium phosphate buffer, pH 7·8.

Sephadex G-50 dextran gel (L. Light and Co. Ltd., Colnbrook, Bucks.) was graded to remove fine particles and washed well by decantation with 5 mM-potassium phosphate, pH 8. The column (prepared from 20 g. of dry gel) was poured as a slurry and allowed to settle with slow flow under gravity.

Diethylaminoethylcellulose floc (DEAE-cellulose) was a gift from Dr E. Lester Smith. It was washed with 2 vol. of N-KOH, followed by water until the supernatant fluid was neutral. Fine particles were removed by repeated decantation in water, and larger particles were rejected by sucking the suspension through glass tubing of 2 mm. internal bore. Columns were prepared from slurries of the graded floc in water and allowed to settle under gravity flow. Potassium phosphate (0·4 M, pH 8·0) was run through the column until the pH of the effluent reached 8·0; this was followed by 20 vol. of 5 mM-potassium phosphate, pH 8·0.

All columns were poured in glass tubes plugged with glass wool; the use of sintered-glass disks produced columns with very low flow rates. Prepared columns were capped with closely fitting disks of Whatman no. 1 filter paper and transferred to a cold room at 0–4°.

Buffers. Phosphate buffers were prepared from KH_2PO_4 and K_2HPO_4 or KOH. Acetate buffers were made from sodium acetate trihydrate and acetic acid, citrate buffers from trisodium citrate dihydrate and citric acid, and borate buffers from $Na_2B_4O_7,10H_2O$ and H_3BO_3.

Chemicals. Tetrahydropteroylglutamic acid was prepared as described by Kisliuk & Woods (1960) and stored as the solid under H_2. Cobalamin was crystalline material (Anacobin) obtained from British Drug Houses Ltd., Poole, Dorset; it was 88% pure by spectrophotometric assay. Adenosine triphosphate, diphosphopyridine nucleotide, reduced diphosphopyridine nucleotide and riboflavin phosphate were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. DL-Homocysteine was from either Mann Research Laboratories Inc., New York, N.Y., U.S.A. or Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Protamine sulphate (from herring roe) was from L. Light and Co. Ltd. Factor B (cobinamide), a form of vitamin B_{12} without a nucleotide residue, was as described by Guest (1960). 5:6-Dimethylbenzimidazolylcobamide coenzyme was a gift from Dr H. A. Barker; in experiments with this compound precautions were taken to exclude light.

Assay of the factor

This was based on the work of Kisliuk & Woods (1960). Determination of the factor content of protein fractions depended on their ability to promote methionine synthesis by extracts of acetone-dried organisms grown without cobalamin in a reaction mixture containing tetrahydropteroylglutamic acid as folic acid cofactor. This system does not respond either to cobalamin itself or to 5:6-dimethylbenzimidazolylcobamide coenzyme.

The standard reaction mixture contained enzyme-containing extract (assay extract) equivalent to 10 mg. of protein and (μmoles in a final volume of 2 ml.): potassium phosphate buffer, pH 7·8, 240; DL-homocysteine, 20; L-serine, 10; hexose diphosphate 15; $MgSO_4$, 10; ATP, 10; DPN, 1; pyridoxal phosphate, 1. In the later part of this work, 0·2 mM-riboflavin phosphate (flavin mononucleotide, FMN) was found to increase the activity of the partially or extensively purified factor by 25–40%, but to be without effect when crude preparations of the factor were used; a requirement for a flavin coenzyme in the synthesis of methionine has been observed with partially purified enzyme fractions from *E. coli* strain 113/3 (Hatch, Takeyama, Cathou, Larrabee & Buchanan, 1959). Consequently, 0·4 μmole of FMN was always added to each assay tube. It was also found that there was a pronounced lag in the synthesis of methionine during the first 30 min. of incubation; this was abolished if reduced DPN were added to the reaction mixture. In a few experiments, $DPNH_2$ (1 μmole/2 ml.) was used in place of DPN.

The reaction mixtures were incubated at 37° for 3 hr. in an atmosphere of H_2 and the reaction was stopped by bringing to 100° for 3 min. After centrifuging, the supernatant fluids were used for the estimation of the methionine formed. This was assayed microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 by the method of Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer, and Kisliuk & Woods (1960) have given information on the specificity of the assay.

Before assaying, fractions containing factor were dialysed for 15 hr. at 3° against 50 mM-potassium phosphate buffer, pH 7·8; those fractions containing Ca^{2+} ions (see below) were dialysed first against 50 mM-potassium phosphate buffer, pH 7·8, containing 5 mM-disodium ethylenediaminetetra-acetate, for 6 hr. and then against 50 mM-potassium phosphate buffer, pH 7·8. Fractions obtained after step IV of the purification were assayed without prior dialysis.

The crude extracts containing factor were themselves able to synthesize methionine to a limited extent, under the conditions used, without the addition of the assay extract. However, a difference curve may be constructed, representing the ability of the factor to promote methionine synthesis by the assay extract (Fig. 1). In practice, the amounts of factor-containing extracts taken for assay were sufficiently small to have negligible ability to synthesize methionine in the absence of assay extract; in effect this meant that only the first portion of the difference curve was used and an almost linear response resulted. On purification, the ability of the preparations to synthesize methionine was rapidly abolished.

The assay for the factor led to rather variable amounts of methionine with any one preparation of the factor, even under strictly controlled conditions; it was therefore considered more satisfactory to define 1 unit of factor activity as that possessed by 1 mg. of protein of the extract prepared from one particular batch of acetone-dried powder by the carefully standardized procedure described above.

Fig. 1. Assay of the factor. Crude factor was incubated for 3 hr. with the assay system. Main source of enzymes (assay extract, see Materials and Methods section) absent, ○; main source of enzymes (equivalent to 10 mg. of protein) present, ●; difference curve, – – –.

Extracts of fresh batches of powder were standardized by direct comparison with the original powder. The units defined are approximately the same as those used by Guest & Woods (1960) in the investigation of synthesis of the factor by ultrasonic extracts of *E. coli*, i.e. 1 unit of factor increases the synthesis of methionine by 10 mg. of protein of the assay extract to the extent of about 100 μm-moles of L-methionine/3 hr.

In each assay of factor activity, a standard curve was constructed over the range 0–5 units, with the crude factor-containing extract of known activity. A response curve of identical shape was given by the factor at all stages of purification. At some steps in the purification the preparations of the factor contained sufficient salts to reduce methionine formation by up to 15%. In such cases the same concentration of the same salts was added to the standards.

Assay of cobalamin

Cobalamin was assayed microbiologically with either *Euglena gracilis* var. *bacillaris* or *E. coli* strain 113/3, a cobalamin- or methionine-requiring auxotroph (Davis & Mingioli, 1950).

With *Euglena gracilis* the procedure adopted was that of Kisliuk & Woods (1960), with the exception that the inoculum was derived from a 4-day culture on basal medium not supplemented with cobalamin; the organisms were centrifuged, resuspended in 3 culture vol. of 0·9% NaCl and 0·1 ml. of this suspension was added to each assay tube. This modified procedure enabled the incubation time to be reduced from 10 to 8 days; it also led to less growth of organisms in tubes devoid of cobalamin. Growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a red filter no. 608.

The method with *E. coli* 113/3 was essentially that of Burkholder (1951). The organism was maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. The basal medium was as described by Burkholder (1951). Tween 80 (Honeywill and Stein Ltd., London, W. 1) was added to a final concentration of 0·01% immediately before using the medium; this reduced the tendency of the organisms to aggregate during growth.

The medium (final volume 2 ml., in 150 mm. × 20 mm. tubes) was autoclaved (7 min. at 115°) after the addition of cobalamin or experimental samples. Standards were included in duplicate in each assay; samples were assayed at three concentrations. The inoculum was derived from a 10 hr. culture on the basal medium containing 0·1 mM-DL-methionine. The organisms were centrifuged, resuspended in the culture volume of water and diluted 50-fold with water; 0·1 ml. of this suspension was added to each assay tube. Incubation was for 15–20 hr. at 37° on a rotary shaking machine (220 rotations/min., radius 2 cm.). Growth was assessed in an EEL colorimeter with a neutral density filter. A reproducible and almost linear curve relating growth to cobalamin concentration was obtained over the range 0–0·6 μmg. of cobalamin/ml. The accuracy of the assay was about $\pm$10%.

As standard, an aqueous solution containing 150 μg. of cobalamin/ml. was assayed spectrophotometrically, the extinction coefficient quoted by Brink *et al.* (1949) being used. This was diluted monthly with KCN solution to give a stock solution containing 1·5 μg. of cobalamin and 1·5 mg. of KCN/ml., and stored at 4°; it was diluted further with water to an appropriate concentration just before use.

Purification of the factor

All manipulations during the purification procedure, including the column chromatography, were done at 0–4°. During the first four steps of the purification particular care was taken to control the pH to within at least $\pm$0·03 unit of the figures quoted below, since even small deviations outside these limits caused a serious decrease in the degree of purification finally achieved. A Pye Type 11087 Dynacap pH meter (W. G. Pye and Co. Ltd., Cambridge), with standard glass electrodes, was suitable for this purpose.

Reagents were added to protein solutions dropwise, with stirring.

Step I; removal of nucleoprotein with calcium chloride. The initial extract of factor, prepared as described earlier, was brought from pH 7·0 to pH 5·65 with N-acetic acid (about 0·7 ml./100 ml. of extract). Material usually precipitated, but redissolved on continued stirring. Calcium chloride (M) was added to a final concentration of 25 mM; the pH fell to 5·35, but rose slowly to 5·55 on stirring for 30 min. The mixture was centrifuged at 24 500*g* for 20 min. and the precipitate discarded.

Step II; removal of protein at pH 5. The supernatant fluid from step I was adjusted to pH 5·00 by the addition of 0·2 M-sodium acetate buffer, pH 4·0 (about 3 ml./100 ml. of initial extract), and stirred for 30 min. The precipitate was removed by centrifuging at 24 500*g* for 20 min. and discarded.

Step III; precipitation of factor at pH 4·4. The supernatant fluid from step II was adjusted to pH 4·40 with N-acetic acid (about 1·9 ml./100 ml. of initial extract) and immediately centrifuged at 10 000*g* for 10 min. The supernatant fluid was discarded and the centrifuge tubes were wiped as free as possible from fluid. The precipitate was dissolved rapidly in 20 mM-potassium phosphate buffer,

pH 7·8 (about 20 ml./100 ml. of initial extract). This solution was adjusted to a protein concentration of 10 mg./ml. by the further addition of 20 mM-potassium phosphate buffer, pH 7·8. The pH was adjusted to 7·0, if necessary, by the addition of a few drops of N-KOH.

Step IV; treatment with protamine sulphate. Residual nucleic acid and some inert protein were removed from the solution from step III with protamine sulphate (20 mg./ml. in 20 mM-potassium phosphate buffer, pH 7·5), 0·55 ml./10 ml. of protein solution being used. After standing for 60 min. with occasional stirring, the mixture was centrifuged at 4000*g* for 15 min. and the precipitate discarded. The supernatant solution could be stored at −15° with only small loss of activity (20% in 1 month). If purification was interrupted at this stage more inert material separated out and could be removed by centrifuging again.

Step V; chromatography of protamine complexes on Celite 545. Supernatant fluid (40 ml.) from step IV was mixed with Celite 545 (1 g.) and treated with the protamine sulphate solution (1 ml./100 mg. of protein). The mixture was allowed to stand for 60 min. with occasional stirring and then poured on to a 2 cm. × 2 cm.² column of Celite 545 in 20 mM-potassium phosphate buffer, pH 7·8. The effluent was caused to run through a second column (40 cm. × 2 cm.²) of Celite 545 prepared in the same way. Both columns were then washed with 20 mM-potassium phosphate buffer, pH 7·8, until the effluent was free from protein; about 150 ml. of buffer was required.

Both columns were washed with 75 mM-potassium phosphate buffer, pH 7·8, until the effluent was again free from protein (about 160 ml. was required). The shorter column was then detached and discarded, and elution of the remaining column continued with 120 ml. of 120 mM-potassium phosphate buffer, pH 7·8. Factor was then eluted from the column with 150 ml. of 160 mM-potassium phosphate buffer, pH 7·8.

The effluent from the columns was collected continuously in fractions of either 5 or 10 ml. with an automatic fraction collector. The flow rate of the columns was usually 30–35 ml./hr., but on a few occasions pressure (1–2 lb./in.²; N_2) was applied to the top of the columns to maintain the flow rate at this value.

Fractions were assayed for activity as soon after delivery as practicable and stored at −15° until the results of the assay were available (about 36 hr.). Fractions were then pooled to give 80 ml. of solution CL, an arbitrary balance being made between the total quantity of factor activity taken and the specific activity.

Step VI; removal of protamine. Solution CL contained protamine, which was removed by pouring slowly through a 2·5 cm. × 2 cm.² bed of CM-70 cellulose in 160 mM-phosphate buffer, pH 7·8; 90 ml. of effluent (CM) was collected.

Step VII; reduction of salt concentration. This was achieved by the gel-filtration technique of Porath & Flodin (1959). A 30 cm. × 4·5 cm.² column of Sephadex G-50 gel was prepared in 5 mM-potassium phosphate, pH 8·0; the void volume (40 ml.) and inner volume (75 ml.) were determined in a control run with a mixture of inert protein and potassium phosphate. Two successive 45 ml. quantities of solution CM were brought to 5 mM-potassium phosphate and 2 vol. of 55 ml. were collected from the column; these were combined to give solution SF.

Step VIII; chromatography on diethylaminoethylcellulose. The factor in SF was loaded on to a 5 cm. × 1·5 cm.² column of DEAE-cellulose, prepared in 5 mM-potassium phosphate, pH 8·0, by allowing the solution to run through under gravity. The column was washed with 50 ml. of 5 mM-potassium phosphate, pH 8·0.

Stepwise elution of the column with increasing, but relatively varying, concentrations of phosphate and chloride was done with 70 ml. portions of three solutions obtained by mixing volumes of 5 mM-potassium phosphate, pH 8·0 (A) and 0·1M-K_2HPO_4 in 0·5M-KCl (B), in the proportions (A:B) of (*a*) 16:3, (*b*) 9:5, (*c*) 9:10. Effluent was collected in fractions of either 5 or 10 ml. with the automatic fraction collector; the smaller fractions were taken when the factor was expected to be eluted. Factor activity was eluted by solution (*c*) as a single peak. Fractions containing factor were pooled to give 20 ml. of solution DF.

Step IX; reduction of salt concentration. This was done with the 30 cm. × 4·5 cm.² column of Sephadex G-50 gel, equilibrated in this case with 50 mM-potassium phosphate, pH 8·0. Effluent (30 ml.), containing purified factor, was collected as solution F and stored at −15°.

RESULTS

Purification of the factor

The overall results of a typical preparation of the purified factor are summarized in Table 1, which shows the specific activity and yield at each step of the procedure.

Table 1. *Purification of a factor containing vitamin* B_{12} *from extract of acetone-dried* Escherichia coli

Stage	Material tested	Volume (ml.)	Total protein (mg.)	Total factor activity (units)	Specific activity (units/mg. of protein)	Overall yield of factor (%)
	Crude extract	127	2530	3210	1·27	100
I	$CaCl_2$ supernatant	110	835	2000	2·39	62
II	pH 5·0 supernatant	108	445	1560	3·51	49
III	pH 4·4 precipitate	27·7	280	1345	4·8	42
IV	Protamine sulphate supernatant	27·5	230	1300	5·6	40
V	Celite 545 eluent, CL	74	1·78	173	97	5·4
VI	CM-70 cellulose eluent, CM	90	1·53	160	104	5·0
VII	First Sephadex gel eluent, SF	110	1·54	160	104	5·0
VIII	DEAE-cellulose eluent, DF	20	0·62	84	136	2·6
IX	Second Sephadex gel eluent, F	30	0·60	93	155	2·9

Acetone-dried organisms. In spite of attempts to produce a uniform starting material by rigid control of the conditions of growth and acetone-drying of the organisms, considerable variation was found in the properties of the acetone-dried powders. Activity varied from 450 to 650 units of factor/g. of acetone-dried powder, though the variation per milligram of extracted protein was somewhat greater (0·7–1·3 unit). Extracts prepared from different powders also showed considerable variation in their content of nucleic acid, as judged by the ratio of the absorption at 280 mμ to that at 260 mμ. These differences are more probably due to unrecognized factors in the acetone-drying procedure rather than in the culturing of the organisms.

Acetone-dried organisms derived from medium to which cobalamin had been added before instead of after autoclaving the other constituents had a much reduced factor content. When the organism was grown with less than the usual 20 μg. of cobalamin/500 ml. of medium, or for 40 hr. instead of 18 hr., the acetone-dried powders had relatively low activity (250–450 units/g.) without any increase in the yield of dried organisms.

Factor B (cobinamide), the analogue of cobalamin with no nucleotide residue, supports the growth of cobalamin auxotrophs of *E. coli* (Ford & Porter, 1953) and is also weakly active in methionine synthesis with ultrasonic extracts of *E. coli* PA15 (Guest, 1960). It was possible that growth on Factor B might yield organisms with increased factor activity since the organism might then synthesize any special form of vitamin B_{12} required; however, replacement of cobalamin in the growth medium by an equal amount of Factor B gave an acetone-dried powder containing only 140 units/g.

Acetone-dried powders of ox liver were also weakly active, containing about 10 units/g. of powder; the specific activity of the extracted protein was about 0·03 unit/mg. and purification of the factor from this source was not therefore undertaken.

Preparation of the extract. Ultrasonic treatment of the aqueous suspension of the acetone-dried powder for a short period gave increased extraction of activity over that obtained by the simpler procedure of Kisliuk & Woods (1960). Although the specific activity of the extract was decreased by 10–30 % by ultrasonic treatment for 75 sec., the yield of extracted material was increased by 1·5- to 2-fold. Treatment for more than 75 sec. resulted in little more activity being extracted and a sharp drop in the specific activity of the extracted material.

Extraction of the powder with acidic or alkaline buffers, or with 1 % cetyltrimethylammonium bromide, was no better than extraction with water. Autolysis or extraction in the presence of butan-1-ol (Morton, 1950) resulted in decreased extraction and specific activity.

Step I; removal of nucleoprotein with calcium chloride. The extract of factor, adjusted to 20 mg. of protein/ml., contained 5–8 mg. of nucleic acid/ml. This was reduced by 70 % in the treatment with $CaCl_2$. The precipitation of nucleic acid was maximum at a final $CaCl_2$ concentration of about 25 mM; the optimum concentration of $CaCl_2$ varied slightly with extracts from different batches of acetone-dried powder and was determined in a pilot experiment with each batch. The procedure also removed 45–70 % of the protein of the extract, with rather variable losses of activity (0–35 %). Considerable and sharp loss of activity occurred if the treatment was carried out at pH values below 5·65, whereas above this value removal of nucleic acid and protein was poor.

Steps II and III; pH *fractionation.* Material precipitated between pH 5·00 and 4·40 contained 10–20 % of the protein of the initial extract and only small amounts of activity were lost in the discarded fractions. Fine control of the pH was necessary for satisfactory fractionation, and prolonged exposure to pH 4·40 resulted in a large loss of activity.

Step IV; treatment with protamine sulphate. The nucleic acid remaining after the $CaCl_2$ treatment was concentrated in the active fraction during steps II and III, but could be almost completely removed by treatment with protamine sulphate at pH 7·0 in 20 mM-potassium phosphate buffer; 15–25 % of the remaining protein was also removed with very small loss of activity.

Step V; chromatography on Celite 545. Addition of an excess of protamine sulphate to the supernatant solution from step IV caused complete precipitation of factor together with a high proportion of the protein. It was possible to extract selectively the factor–protamine complex from the precipitated material with phosphate buffer, pH 7·8, and to chromatograph this on a Celite 545 column. The most satisfactory procedure was that described in the Materials and Methods section, and the results of such a chromatogram are shown in Fig. 2*a*.

The sequence of events on the columns was deduced from a study of protein, protamine and factor distributions obtained when steps in the procedure were omitted, the formation of a precipitate on dialysis overnight against 20 mM-potassium phosphate, pH 7·8, being used as an indication of the presence of protamine.

Addition of protamine sulphate to the step IV supernatant produced a solid phase containing protamine, factor and inert protein, with excess of protamine and inert protein in the liquid phase.

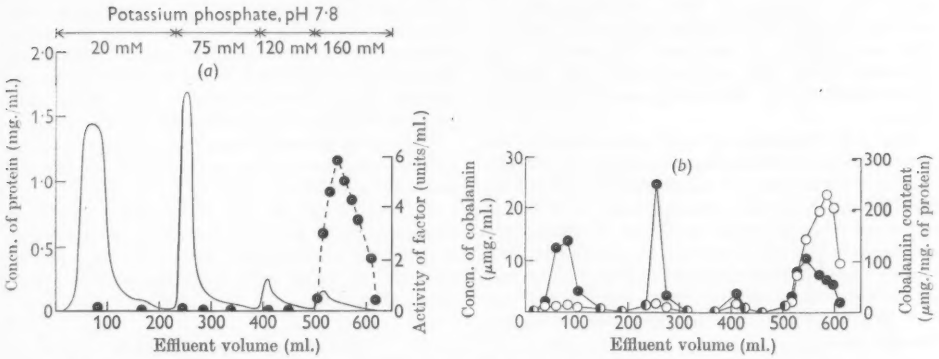

Fig. 2. Chromatography of step IV supernatant fluid on Celite 545. The solution (40 ml.; 27 units/ml.; specific activity, 5·0 units/mg. of protein) was chromatographed as described in the Materials and Methods section. (*a*) Distribution of protein and factor. Protein (—) was estimated for each fraction; points are omitted for clarity. Factor concn., ●- - ●. (*b*) Distribution of vitamin B_{12}. The vitamin B_{12} content of fractions was measured directly with *E. coli* 113/3 and expressed as μmg. of cobalamin/ml. (●—●) or μmg. of cobalamin/mg. of protein (○).

Before forming the solid phase into a short column a little Celite 545 was used to disperse the protein particles into a bed through which buffer solution could percolate freely. The liquid phase was washed (with 20 mM-potassium phosphate, pH 7·8) through the longer column of Celite 545. This column adsorbed the excess of soluble protamine but very little of the inert soluble protein. Some inert protein and all the factor–protamine complex of the solid phase in the shorter column could be brought into solution with 75 mM-potassium phosphate, pH 7·8; as this ran through the longer column, factor was selectively adsorbed on the protamine-coated Celite 545. The inactive shorter column was then discarded and some inert material washed out of the longer column with 120 mM-potassium phosphate, pH 7·8. The factor–protamine complex was completely eluted with 160 mM-potassium phosphate, pH 7·8.

The bulk of the factor was eluted from the column slightly behind the peak of protein washed out with the 160 mM-potassium phosphate (Fig. 2*a*), and it was necessary to determine its exact location for each chromatogram. Since the factor became increasingly unstable to storage after purification beyond step IV, it was necessary to determine the factor distribution as rapidly as possible. The routine quantitative assay required 3 working days, but an adequate qualitative picture could be obtained by reading the methionine assay with *L. mesenteroides* P 60 after growth for only 18 hr. Alternatively the distribution of vitamin B_{12} activity for *E. coli* 113/3 was used (see below).

The combined active fractions had a specific activity of 80–110 units/mg. They accounted for only 13–60 % of the total activity derived from step IV, the remainder being lost by inactivation during the procedure.

This step could also be performed with a single column of Celite 545 or Celite 535 and a continuous concentration gradient of potassium phosphate buffer. This sometimes gave up to 95 % recovery of activity, with purified material occasionally of specific activity as high as 150 units/mg. However, there was very wide variation (15-fold) in the specific activity obtained and this method was abandoned.

Steps VI and VII; removal of protamine and reduction of salt concentration. Before reduction of the phosphate content of the purified fraction from step V, protamine was conveniently removed by treatment with CM-70 cellulose; 14 % of the protein was removed in this way with negligible loss of activity. Reduction of the phosphate concentration to 5 mM was rapidly achieved with the Sephadex gel-column technique without the loss of activity found with the alternative dialysis procedure.

Step VIII; chromatography on diethylaminoethylcellulose. The protein from step VII was held completely by DEAE-cellulose equilibrated with 5 mM-potassium phosphate, pH 8·0, and could be eluted completely as three separate peaks with a continuous gradient up to 0·1 M-K_2HPO_4–0·5 M-KCl. Only the third peak had factor activity. A stepwise gradient was always used to develop the chromatogram (Fig. 3) since this gave sharper

peaks. The active peak had a constant specific activity of about 130 units/mg. of protein (Fig. 4). Only about 50% of the step VII activity was recovered from the column; this was mainly attributable to the increasing instability of the factor.

Step IX; reduction of salt concentration. The active solution DF from chromatography on DEAE-cellulose contained 55 mM-potassium phosphate and 263 mM-KCl; this concentration of salt was sufficient to cause some inhibition of methionine synthesis in the assay system for the factor. The treatment with Sephadex gel exchanges this salt for 50 mM-potassium phosphate, pH 8·0, without any loss of protein and a slight increase in the specific activity.

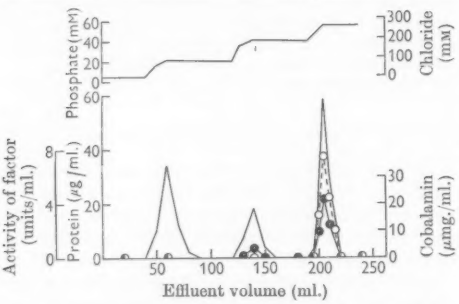

Fig. 3. Chromatography of solution SF on DEAE-cellulose. The solution (110 ml.; 1·45 unit/ml.; specific activity, 104 units/mg. of protein) was chromatographed as described in the Materials and Methods section. The solvent gradient was determined by estimation of the effluent phosphate content according to Fiske & Subbarow (1925). Protein (—) was estimated for each fraction; points are omitted for clarity. Factor concn., ○. The vitamin B_{12} content of fractions was measured directly with *E. coli* 113/3 (●).

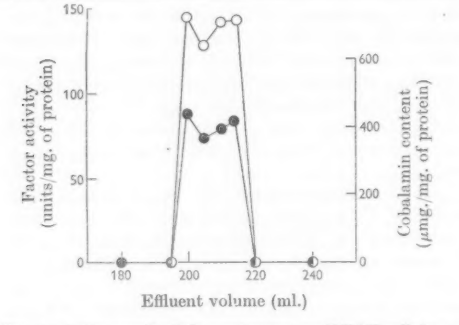

Fig. 4. Active peak of chromatogram on DEAE-cellulose. Conditions were as described for Fig. 3. Specific activity of factor, ○. Vitamin B_{12} content, ●.

The purified factor solution F contained only 3% of the activity extracted initially from the acetone-dried organisms. The specific activity of the product was always 145–155 units/mg., although derived from different batches of acetone-dried powders showing considerable variation in properties.

The ultraviolet-absorption spectrum of the purified preparation has a maximum at 275 mμ and a 280 mμ/260 mμ ratio of 1·6.

At each stage of the purification procedure combinations of fractions were assayed for factor activity but no evidence was found of the resolution of the factor into two components acting synergistically.

Stability of the purified factor

Acetone-dried organisms, crude extracts or preparations purified as far as the end of step IV, retained their activity during storage at −15° for at least 3 months. However, preparations taken beyond step IV became increasingly unstable, the final product having a half-life of about 3 days at −15° (Table 2). Storage in the presence of glutathione, cysteine, 2-mercaptoethanol, potassium ascorbate (all at 5 mM) or disodium ethylenediaminetetra-acetate (1 mM) did not improve the stability. Attempts to concentrate the factor solution (by desalting with Sephadex gel and freeze-drying) in the hope of increasing the stability to storage, resulted in considerable denaturation and loss of activity with the formation of an orange amorphous powder.

Kisliuk & Woods (1960) reported that the factor present in crude extracts was destroyed by heating. This thermolability was shown by the purified factor to an increased extent (Fig. 5). Crude preparations of the factor were reasonably stable to both acid and alkali, but the purified factor showed a sharp pH optimum for stability at pH 8·0–8·5 (Fig. 6).

Factor present in crude extracts or purified by steps I–IV was completely non-diffusible. Dialysis of more purified preparations resulted in loss of

Table 2. *Stability of factor to storage*

Solutions of factor-containing fractions of known activity were stored at −15° for periods varying from 2 days to 3 months. Day-to-day variations in the amounts of methionine formed in the assay system permit only approximate estimations of the half-life of the factor during purification.

Fraction	Half-life of factor activity
Acetone-dried organisms; crude extract	> 3 months
Step IV supernatant	3 months
Step V solution CL	9 days
Step VII solution SF	6 days
Step IX solution F	3 days

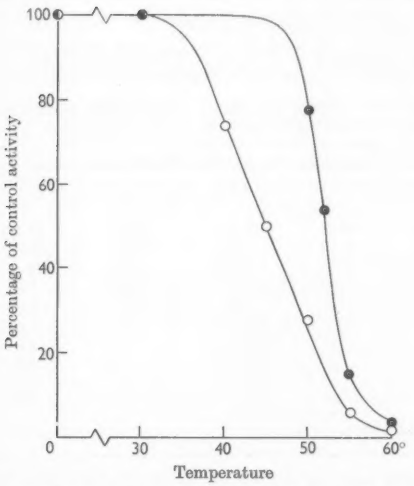

Fig. 5. Effect of heat on the factor. Samples (3 ml.) of crude factor (specific activity, 1·8 unit/mg. of protein; 60 units/ml. of 50 mM-potassium phosphate buffer, pH 7·8) or purified factor (specific activity, 143 units/mg. of protein; 5 units/ml. of 50 mM-potassium phosphate buffer, pH 8·0) were heated rapidly from 0°, maintained for 5 min. at a fixed temperature and cooled rapidly to 0°. Results are expressed as percentage of factor activity of control tube maintained at 0°. Crude factor, ●; purified preparation, ○.

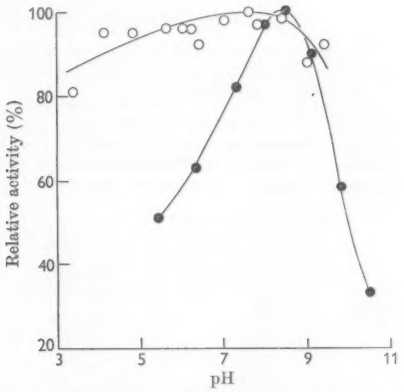

Fig. 6. Effect of pH on the stability of the factor. Samples (1·8 ml.) of crude factor (specific activity, 2·3 units/mg. of protein; 23·5 units/ml. of 50 mM-potassium phosphate, pH 7·8) were adjusted to the pH shown by addition of 0·2M-sodium borate (pH range 7·6–9·4), 0·2M-potassium phosphate (pH range 6·1–7·8) or 0·2M-sodium citrate (pH range 3·4–6·2). Samples (2·5 ml.) of purified factor (139 units/mg. of protein; 3·6 units/ml. of 50 mM-potassium phosphate, pH 8·0) were adjusted to the pH shown with acetic acid or KOH. After keeping for 40 min. at 0° the pH was checked and readjusted to 7·8 with 0·5M-potassium phosphate, pH 7·8, before assay of factor. Results are expressed as a percentage of the maximum activity; crude factor, ○; purified preparation, ●.

activity. For example, dialysis of solution CM from step VI against 5 mM-potassium phosphate, pH 8·0, at 0° for 12 hr. caused 60% inactivation; dialysis of solution DF from step VIII against the same buffer at 0° for 15 hr. resulted in 90% inactivation. Since there was no concurrent loss of vitamin B_{12} activity, the loss of factor activity in these cases is more probably a reflexion of the increased lability of the purified material to heat and storage, rather than to the factor having become diffusible. Addition of cobalamin, 5:6-dimethylbenzimidazolylcobamide coenzyme or extract of heated *E. coli* (prepared according to Guest, 1960) did not restore the activity of purified preparations partially inactivated by dialysis.

Cobalamin content of the factor

Assays for vitamin B_{12} with either or both *Euglena gracilis* and *Escherichia coli* 113/3, were performed on all the fractions produced by the purification procedure. There was little correlation between vitamin B_{12} activity and factor content of fractions obtained in steps I–IV (Table 3); there were also variable losses of vitamin B_{12} on dialysis, under conditions where there was no loss of factor.

However, examination of the distribution of vitamin B_{12} in the fractions obtained in the Celite 545 chromatogram showed that, although all the protein-containing fractions contained vitamin B_{12}, the peak of factor activity was associated with protein containing a much higher proportion of vitamin B_{12} (Fig. 2, compare *a* and *b*). With cyanocobalamin as standard, the same picture of vitamin B_{12} distribution was obtained with either assay organism and was not altered by prior dialysis of the fractions against 50 mM-potassium phosphate, pH 7·8. Inactive protein from the column contained 5–20 μmg. of cobalamin/mg. of protein, and the active peak contained up to 230 μmg./mg. of protein.

The cobalamin content of the active material was not reduced by treatment with CM-70 cellulose (step VI) or with Sephadex gel (step VII). This latter treatment would be expected to remove any vitamin B_{12} activity due to relatively small molecules, such as free cobalamin or simple derivatives of it.

Vitamin B_{12} activity was almost entirely associated with factor activity in the DEAE-cellulose chromatogram (Fig. 3). The third peak had uniform proportions of protein, factor and vitamin B_{12}

Table 3. *Cobalamin content of the factor at various stages of purification*

The cobalamin content of fractions was assayed with *Euglena gracilis* or *Escherichia coli* 113/3. Where indicated, samples were first dialysed as detailed in the Materials and Methods section for the assay of factor activity: +, dialysed; −, not dialysed.

Assay organism	Purification step	Dialysis	Cobalamin content (μmg./mg. of protein)		Factor content (units/mg. of protein)		Cobalamin/factor (μmg./unit)	
			Precipitate	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant
Euglena gracilis	Crude extract	+	.	14·5	.	1·15	.	12·6
	I	+	7·3	16·6	0·25	1·45	29·0	11·4
	II	+	11·0	17·8	1·10	1·55	10·0	11·5
	III	+	20·5	16·7	3·35	0·02	6·1	835
	IV	+	7·6*	29·6	1·30*	4·70	5·8*	6·3
Escherichia coli 113/3	IV	+	.	16·8	.	5·0	.	3·4
	V	−	.	185	.	105	.	1·8
	VI	−	.	200	.	105	.	1·9
	VII	−	.	200	.	104	.	1·9
	VIII	−	.	400	.	130	.	3·1
	IX	−	.	425	.	143	.	3·0

* Calculated by difference.

Table 4. *Cobalamin content of the purified factor*

Cobalamin assays were with samples of purified factor with a specific activity 143 units/mg. of protein. Samples were (i) autoclaved directly in growth medium, (ii) added unheated to autoclaved medium (after treatment with 2 vol. of ethanol and sterile dilution for the *Euglena gracilis* assay), (iii) digested for 24 hr. at 27° with crystalline papain (1 mg./mg. of protein of factor) in 0·25 M-potassium phosphate, pH 8·0, containing 1 mM-disodium ethylenediaminetetra-acetate and 5 mM-cysteine, and (iv) heated at 100° for 30 min. in a sealed tube with 0·1 M-sodium acetate buffer, pH 4·5, containing 0·1 mM-KCN.

Treatment of samples	Cobalamin content of factor (μmg./mg. of protein) assayed with	
	Escherichia coli 113/3	*Euglena gracilis*
(i) Autoclaved in medium	425	550
(ii) Not autoclaved in medium	94	550
(iii) Digested with papain	495	510
(iv) Extracted with HCN	575	560

activity (Fig. 4). These results are strong evidence that the factor is a derivative of vitamin B_{12}.

The cobalamin content of fractions was usually determined after autoclaving a sample in the growth medium of the assay organism (i.e. at pH 6·8 or 4·5). If the factor was assayed (*E. coli* 113/3) unheated, the apparent cobalamin content was only one-quarter of that when it was heated in the medium (Table 4). Presumably the vitamin B_{12} bound in the factor is largely, or even completely, unavailable to *E. coli* 113/3, but is rendered available by heating.

The purified factor was also digested with crystalline papain; this treatment released slightly more vitamin B_{12} than autoclaving (Table 4). After heating the factor at 100° with HCN the *E. coli* 113/3 assay showed a still higher value (Table 4).

With the *Euglena gracilis* assay treatment of the solution DF with 2 vol. of ethanol before sterile dilution and assaying without heating gave the same cobalamin content as that obtained by heating in the medium (Table 4). This may represent release of vitamin B_{12} activity either by the ethanol or during the long growth period of *Euglena gracilis* rather than reflecting the activity of the native factor for this organism. Digestion with papain or heating with HCN as described above gave essentially similar cobalamin values with *Euglena gracilis* (Table 4).

The crude extracts of acetone-dried organisms contained 15 μmg. of non-diffusible cobalamin/mg. of protein, assayed with *Escherichia coli* 113/3; no attempt was made to release bound vitamin B_{12} except by the autoclaving of the samples in the growth medium. Thus the factor can account for only 16% of the bound cobalamin of the initial extracts. This result is to be expected since the organisms were grown in the presence of a higher concentration of cyanocobalamin than is required to satisfy the vitamin B_{12} requirement of *E. coli* auxotrophs (Davis & Mingioli, 1950).

Further properties of the factor

Effect of proteolytic enzymes. Digestion of the purified factor with crystalline papain caused complete loss of activity (Table 5). Similar results were obtained with crystalline trypsin.

Effect of cyanide. Factor purified as far as the end of step V was exposed to various concentrations of cyanide, which was subsequently removed

Table 5. *Digestion of purified factor by papain and trypsin*

In Expt. 1 a sample (3 ml.) of purified factor (20 μg. of protein/ml.; specific activity 155 units/mg. of protein) was digested for 3 hr. at 25° with crystalline papain (10 μg./ml.) in 50 mM-potassium phosphate, pH 8·0, containing 1 mM-disodium ethylenediaminetetra-acetate and 5 mM-cysteine. In Expt. 2 a second sample (3 ml.) was digested for 1 hr. at 25° with crystalline trypsin (20 μg./ml.) in 50 mM-potassium phosphate, pH 8·0. Samples (0·6 ml.) of digested factor were taken for assay. Controls were (i) samples incubated in the absence of proteolytic enzyme, and (ii) and (iii), proteolytic enzyme added in the assay of the factor to tubes with and without 0·6 ml. of factor solution. All values are corrected for a L-methionine content (30 μm-moles) of a tube, containing the digested factor and the assay system, heated at 100° for 3 min.

Expt.	Addition to assay system	Total L-methionine formed (μm-moles/3 hr.)	L-Methionine formed corrected for that formed by proteolysis during factor assay (μm-moles/3 hr.)
1	Papain-digested factor	63	6
	Undigested factor	238	238
	Undigested factor + papain	277	220
	Papain alone	57	.
2	Trypsin-digested factor	88	10
	Undigested factor	274	274
	Undigested factor + trypsin	332	254
	Trypsin alone	78	.

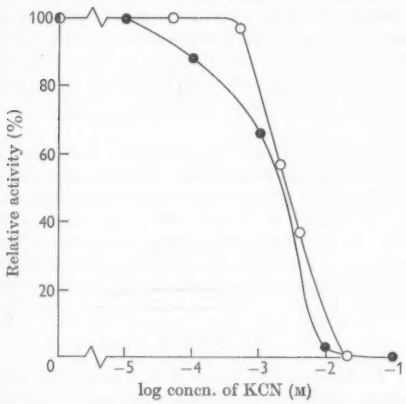

Fig. 7. Effect of cyanide on the factor. In Expt. 1 (●) samples (3·0 ml.) of purified factor (specific activity, 135 units/mg. of protein; 5·4 units/ml. of 50 mM-potassium phosphate, pH 8·0) were treated with KCN (brought to pH 8·0 with HCl) at 22° for 30 min. Cyanide was removed by dialysis for 13 hr. against five changes of 50 mM-potassium phosphate, pH 8·0, before assay of factor. In Expt. 2 (○), the KCN was added directly to the assay system for the factor.

by dialysis. Loss of activity varied with the concentration of cyanide (Fig. 7), 2·2 mM-KCN causing 50% inactivation. Similar concentrations of KCN also inhibited the activity of the factor in catalysing the synthesis of methionine by the assay system; 2·5 mM-KCN was required for 50% inhibition (Fig. 7).

Effect of light. Irradiation for 1 hr. of a purified factor preparation (containing 157 units/mg. in 0·1 M-potassium phosphate, pH 8·0) with light from a 100w tungsten lamp, placed 25 cm. distant, was without effect on the activity. When irradiated in a silica cuvette placed 25 cm. in front of a u.v. lamp (7w, principal emission at 2537 Å), the solution lost 67% of its activity compared with a non-irradiated control sample. This loss of activity, or that caused by KCN, was not restored by cobalamin, 5:6-dimethylbenzimidazolylcobamide coenzyme or an extract of heated organisms.

Charcoal treatment. No activity was lost by the most purified preparation (solution F) when a sample (3 ml.) was stirred for 20 min. with 1·5 mg. of ground and acid-washed Nuchar-C activated charcoal (West Virginia Pulp and Paper Co., New York, N.Y., U.S.A.) and the charcoal removed by centrifuging.

Treatment of crude extracts of the factor with activated charcoal according to the method of Barker *et al.* (1960*a*) caused 27% inactivation.

DISCUSSION

The factor when purified 150-fold is non-diffusible and unstable to heat; it also shows an instability towards both acid and alkali, being most stable in the pH range 8·0–8·5. Treatment of the purified factor with the proteolytic enzymes papain and trypsin causes complete loss of activity. These facts, together with the 280 mμ/260 mμ absorption ratio of 1·6, indicate that the factor activity is associated with a protein. Preparations of ribonucleic acid made from the crude extracts by the method of Kirby (1956) had no activity.

The factor can promote the formation of at least five times its own weight of L-methionine in the assay system used and so, on the basis of the

definition given by Dixon & Webb (1958), it is probably an enzyme concerned in the synthesis of methionine by the system studied.

The factor responds to purification techniques which have been applied to enzymes, e.g. isoelectric precipitation and chromatography on DEAE-cellulose. Less efficient purification could also be obtained by fractionation with ammonium sulphate or acetone, or by selective adsorption on calcium phosphate or alumina Cγ gels (unpublished observations). The use of calcium chloride to remove nucleoprotein from the crude extracts is a novel procedure which was found preferable to the use, at this stage, of protamine sulphate, manganese chloride or cetyltrimethylammonium bromide. The chromatography of the factor on Celite 545 with protamine sulphate is also novel. Partition chromatography of enzymes on Celite adsorbents has usually met with little success (Porter, 1955), but a similar procedure to that described here has been used successfully in the fractionation of deoxyribonucleic acids (Brown & Watson, 1953).

The instability of the purified factor has so far prevented the production of preparations sufficiently concentrated to permit studies on its homogeneity or the investigation of its absorption spectrum in the visible region.

The present results provide evidence that vitamin B_{12} activity is closely associated with the factor, i.e. that the latter is a vitamin B_{12}-containing enzyme. The form in which vitamin B_{12} is present is not yet known. Relative to cyanocobalamin, the intrinsic vitamin B_{12} content is largely unavailable for *E. coli* 113/3 but is present in some combined form from which it can be released by heating, papain digestion or extraction with hydrogen cyanide. As well as facilitating the extraction of vitamin B_{12}, hydrogen cyanide probably protects it during autoclaving by converting it into the stable cyano forms (Ford, 1953). The intrinsic vitamin B_{12} of the factor may be completely available to *Euglena gracilis*. The material obtained after autoclaving at pH 4·5 is as effective for this organism as that released by papain digestion or cyanide extraction.

The minimum measured vitamin B_{12} content of the factor (1·9 μmg. of cobalamin/unit) corresponds to a 'turnover number' of the vitamin in methionine synthesis of about $4{\cdot}6 \times 10^2$ moles of L-methionine/mole of cobalamin/min. in the factor assay system. However, the significanse of this value is dubious since (*a*) the true vitamin B_{12} content may not be that measured on the assumption that the vitamin is present as cobalamin, and (*b*) the absolute amounts of L-methionine produced in the factor assay system vary for any given amount of factor.

Several coenzyme forms of the cobalamins have been obtained from *Clostridium tetanomorphum* and *Propionibacterium shermanii* for the formation of β-methylaspartic acid from glutamic acid in cell-free extracts (Weissbach, Toohey & Barker, 1959; Barker *et al.* 1960*a*, *b*). A coenzyme form of vitamin B_{12} has also been shown to act in the isomerization of succinyl-coenzyme A to methylmalonylcoenzyme A by cell-free extracts of *P. shermanii* (Stadtman, Overath, Eggerer & Lynen, 1960) or of mammalian tissues (Gurnani, Mistry & Johnson, 1960; Stern & Friedman, 1960; Marston, 1960). Guest & Woods (1960) found that the 5:6-dimethylbenzimidazolylcobamide coenzyme was more effective than cobalamin in the synthesis of the present factor by ultrasonic extracts of *E. coli* PA 15, but it would not replace the activity of the factor in promoting methionine synthesis in the presence of tetrahydropteroylglutamic acid. It seems possible that the factor is an enzyme containing a cobamide coenzyme (or a derivative) as prosthetic group.

In support of this hypothesis, the factor is destroyed by ultraviolet light and inactivated by cyanide ion, as are the cobamide coenzymes. However, it is not affected by light from a tungsten lamp, nor by treatment with activated charcoal as one might expect from the results of Barker *et al.* (1960*a*).

No evidence has been presented here for the nature of the function of the factor in methionine synthesis. The possibilities have been discussed fully by Kisliuk & Woods (1960) and Guest *et al.* (1960). It is difficult to envisage a mechanism for the action of cobalamin and its derivatives common to all the enzyme systems *in vitro* which have been studied.

SUMMARY

1. *Escherichia coli* strain PA 15 contains, when grown in the presence of cobalamin, a factor which enables tetrahydropteroylglutamic acid to serve as the C_1-transfer cofactor for methionine synthesis by extracts of acetone-dried organisms grown without cobalamin. A method is described for the assay of this factor and the preparation of extracts rich in it.

2. The purification (150-fold) of the factor is described. It includes removal of nucleoprotein with calcium chloride, pH fractionation, column chromatography on Celite in the presence of protamine and chromatography on diethylaminoethylcellulose.

3. The factor is non-diffusible and when purified is unstable to heating and to storage even at low temperatures. It is most stable at pH 8·0–8·5.

4. The protein nature of the factor is indicated by the 280 mμ/260 mμ absorption ratio of 1·6 and the loss of activity on digestion with trypsin and papain.

5. During the later stages of purification the factor is closely associated with bound vitamin B_{12}. Chromatography on diethylaminoethylcellulose produces an apparently homogeneous peak with a constant ratio of factor:protein:vitamin B_{12}.

6. The vitamin B_{12} of the factor is largely unavailable for *Escherichia coli* strain 113/3 (a cobalamin or methionine auxotroph) but can be released by heating, digestion with papain or extraction with hydrogen cyanide.

7. The purified factor is destroyed by cyanide. Irradiation with ultraviolet light inactivates the factor, but irradiation with visible light or treatment with activated charcoal is without effect.

8. Since the factor is a protein and it acts catalytically and contains vitamin B_{12}, it is suggested that it is an enzyme with a prosthetic group related to cobalamin and concerned in the conversion of homocysteine into methionine under the conditions studied.

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Differences in Sodium and Potassium Loss by Kidney Mitochondria

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In a study of ion transport by rat-heart and skeletal-muscle mitochondria, Ulrich (1959) presented evidence that the potassium, but not the sodium, gradient maintained by these particles could be increased by metabolic activity during incubation at 37°. Bartley & Davies (1954) reported that sheep-kidney mitochondria transported both of these ions against concentration gradients when adenosine triphosphate and an oxidizable substrate were present in the incubating medium. Since it was of some interest to determine the cause for these differences, some of the experiments of Bartley & Davies were repeated with rabbit-kidney mitochondria. Their findings with respect to sodium transport could not be confirmed. The results demonstrate that the sodium gradient maintained by rabbit-kidney mitochondria cannot be increased by metabolic activity, and that in several other respects the transport of these two ions by kidney mitochondria is different. A preliminary account has been given (Ulrich, 1961).

METHODS

Chemicals. Adenosine triphosphate (disodium or potassium salt; ATP) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. Adenosine 5′-phosphate (sodium salt; AMP) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. α-Oxoglutaric acid (A grade) was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Succinic acid (Fisher certified reagent) and fumaric acid (Fisher purified) were obtained from the Fisher Scientific Laboratory, New York. L-Glutamic acid was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. 2:4-Dinitrophenol was obtained from the Eastman Kodak Co., Rochester, New York. Sucrose was analytical grade.

Preparation of mitochondria. Mitochondria were isolated from the kidneys of white, New Zealand strain, male rabbits (about 3 kg. body wt.). All operations were carried out at 2–4°. The animals were stunned by a blow at the back of the neck and the kidneys were rapidly removed and plunged into 0·25M-sucrose solution at 0°, which was prepared not more than 18 hr. before the beginning of the experiment. The capsule was removed and, after each kidney had been sagittally divided into halves, the kidneys were transferred twice to fresh 0·25M-sucrose solutions, blotted on filter paper to remove excess of sucrose and then minced with scissors. Portions (5 g.) of mince were homogenized with a size C Teflon model homogenizer (Arthur H. Thomas Co.) for approx. 1 min. (17 complete strokes of the homogenizer) with 35 ml. of 0·25M-sucrose. Sucrose (10 ml.) was then added to give a 10% (w/v) homogenate. The homogenate was centrifuged at 600*g* in a PR-2 International refrigerated centrifuge at 0° for 5 min. The resulting supernatant was filtered through two layers of water-washed no. 120 Curity-grade cheese-cloth (Kendall Co., Walpole, Mass.) and then centrifuged for 10 min. at 7600 rev./min. (5000*g*) in the no. 30 rotor of the Spinco model L preparative ultracentrifuge. The mitochondria were washed twice with approx. 3 ml. of 0·25M-sucrose/g. of mince, first by resuspending the mitochondria with a homogenizer and centrifuging for 10 min. at approx. 16 700 rev./min. (25 000*g*), and secondly by resuspending with a stirring rod and again centrifuging at 25 000*g* for 10 min. The surface of the mitochondrial pellets was also rinsed after each centrifuging with small amounts of the sucrose solution to remove as much of the fluffy layer as possible. After the second washing, the centrifuge tubes were drained, the inside walls wiped with filter paper and the mitochondria immediately suspended in the appropriate solution.

Solutions. All solutions were prepared with water redistilled with Pyrex glassware. Solutions containing an adenine nucleotide and oxidizable substrate were adjusted to pH 7·4 with solid tris (2-amino-2-hydroxymethylpropane-1:3-diol). The same amount of tris was added to the control solution and the pH of this solution was then adjusted to 7·4 with 5N- and 1N-HCl. A Beckman pH meter, model G, with glass electrode, standardized with Beckman buffer solution, pH 7, was used for all pH measurements.

Nomenclature. Suspending media refer to the solutions with which the mitochondria were suspended. Incubating media refer to the solutions to which the mitochondrial suspensions were added immediately before starting the incubation. Adenine nucleotides, oxidizable substrates and inhibitors were added only to the incubating media.

Incubation of mitochondria. In experiments with the 8–10 medium (see next paragraph), 2 ml. of the mitochondrial suspension at 0° were added to 2 ml. of incubating medium in a 20 ml. Pyrex beaker, the incubating medium having first been warmed to 37°. In experiments with the 0–1 medium (see next paragraph), 0·5 ml. of the suspension was added to 3·5 ml. of incubating medium. The 4 ml. suspensions were incubated with gentle shaking in air at 37° in a Dubnoff metabolic incubator. Isolation of the mitochondria after incubation, determinations of mitochondrial wet and dry weights and analyses of sodium and potassium were carried out as described previously (Ulrich, 1959).

Changes in sodium and potassium gradients. Two kinds of experiments were performed: (*a*) Mitochondria were sus-

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pended in a solution containing 8–10 mM-sodium or potassium or both. The suspension was then added to a solution containing approximately the same amount of sodium and potassium and incubated at 37°. These are referred to below as experiments with the 8–10 medium. (*b*) The mitochondrial suspension was added to a solution containing either no sodium and potassium or else only very small amounts (approx. 1 mM) of these ions. These are referred to below as experiments with the 0–1 medium. Experiments were performed with the 0–1 medium to obtain considerably steeper sodium and potassium gradients than would have been possible with the 8–10 medium.

In the present study, the mitochondrial sodium and potassium content was measured only at the end of an incubation period. Therefore changes in the ion contents and in the ion gradients maintained by the particles are undoubtedly due to changes in the rate of loss of sodium and potassium from the mitochondria. Experiments (unpublished) have shown that if twice-washed rat-liver mitochondria are suspended in a solution at 0° containing 8–10 mM-potassium, the potassium gradient maintained by the particles is between 7 and 8. However, if the ice-cold suspension is added to medium at 37° with the same potassium concentration, and incubated for 20 min., the potassium gradient decreases to 2·6. Addition of ATP and succinate to the incubating medium increases the gradient to 4·6. Further, the results in Table 2 indicate that as the period of incubation is increased, increasing amounts of potassium and, to a less extent, sodium leak out of the particles. These experiments indicate that the mitochondria are not in a steady state with regard to their sodium or potassium contents when incubated at 37°.

RESULTS

Experiments with the 8–10 *medium.* Table 1 shows results from experiments with two different mitochondrial preparations in which ATP and a number of oxidizable substrates were added to the incubating medium. In the first experiment, unwashed mitochondria incubated for 20 min. without ATP and substrate maintained lower sodium and potassium gradients than did twice-washed mitochondria. This appeared to be due to the greater swelling of the unwashed mitochondria, since all data for sodium and potassium were calculated from the mitochondrial water content, i.e. the difference between the wet and dry weight. Addition of ATP and α-oxoglutarate to the medium significantly increased the concentration gradient of potassium but had little effect on the sodium gradient. Similar results were obtained in the second experiment, in which succinate or fumarate was used. The sodium gradient was decreased with ATP and substrate, but this change was small.

Werkheiser & Bartley (1957) have shown that at 0° the percentage dry weight of mitochondria prepared with 0·25 M-sucrose at pH 5–6 was significantly less than that of particles prepared with sucrose at pH 7·8. They stated that examination of the pH 5–6 sucrose mitochondria in the phase-contrast microscope showed the particles to be swollen, whereas mitochondria prepared with the pH 7·8 sucrose appeared unswollen. Pauly, Packer & Schwan (1960) reported that rat-liver mitochondria isolated with the usual acid sucrose solution appeared small, dark and shrunken in the phase-contrast microscope. Since our inability to demonstrate an increase in the sodium gradient might have been due to the use of swollen (and thus damaged?) mitochondria, the mitochondria were isolated with the alkaline sucrose solution. The sucrose solution, adjusted to pH 7·8 with 0·2 M-tris,

Table 1. *Effects of adenosine triphosphate and substrates on the loss of sodium and potassium from rabbit-kidney mitochondria*

Unless otherwise indicated, in this and subsequent Tables mitochondria were washed twice with 0·25 M-sucrose as described in the text. All figures are the means of three incubations ± S.E. Where only two incubations were carried out, the figures for both are presented. The mitochondria were suspended with the following solution: 5 mM-NaH_2PO_4,H_2O–Na_2HPO_4 (pH 7·4), 5 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), 0·25 M-sucrose, 5 mM-$MgSO_4$. A portion (2 ml.) of the mitochondrial suspension was added to 2 ml. of incubating medium with the same composition together with or without 4 mM-substrate, 1 mM-sodium ATP and 1 mM-potassium ATP. The mitochondria were incubated in air for 20 min. at 37°.

Expt. 1	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
Unwashed control	19·2±0·15	12·7±0·3	1·68±0·05	14·7±0·8	1·69±0·13
Unwashed, ATP + α-oxoglutarate	20·3±0·1	19·2±1·1	2·29±0·14	16·5±1·2	1·57±0·10
Control	28·8±0·0	16·2±0·4	2·19±0·09	17·1±0·7	2·15±0·13
ATP + α-oxoglutarate	28·4±0·2	25·2±0·8	2·89±0·11	21·6±2·0	2·36±0·64
Expt. 2					
Control	25·3±0·1	13·7±0·2	1·65±0·02	15·7±0·2	1·94±0·02
ATP + succinate	24·2±0·2	26·2±0	2·82±0	17·1±0·5	1·73±0·05
Control	24·1±0·3	14·6±0·1	1·76±0·01	16·2±0·1	2·00±0·01
ATP + fumarate	24·9±0·4	27·0±0·1	2·90±0·01	16·9±0·1	1·70±0·03

was used approx. 18 hr. after preparation, at which time the pH had fallen to 7·3. The results (Table 2) show that the loss of sodium from the mitochondria was not consistently or significantly changed with the addition of ATP and α-oxoglutarate to the incubating medium. As the incubation time was increased from 5 min. to 1 hr., the control potassium gradients decreased, until at the end of 1 hr. the gradient was approximately half the 5 min. value. The control sodium gradients decreased only 16 % after 1 hr.

The effect of ATP and α-oxoglutarate in increasing the percentage dry weight of the mitochondrial pellets was least after 5 min. incubation and greatest after 60 min. Although the amount of fluffy layer appeared to be somewhat less with the alkaline sucrose, the movements of sodium and potassium and the percentage dry weight of the particles were similar to those of mitochondria prepared with the acid sucrose. Comparison with Werkheiser & Bartley's results cannot be made because in our experiments mitochondrial water was determined only after a period of incubation at 37° instead of at 0°. Further, in the present experiments the media contained not only sucrose but other compounds as well.

Table 2. *Loss of sodium and potassium from rabbit-kidney mitochondria isolated in an alkaline sucrose solution*

Conditions were the same as described in Table 1 except that the mitochondria were isolated in 0·25 M-sucrose, pH 7·3.

Incubation time (min.)	Additions	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
5	None	25·4±0·4	25·7±0·2	3·09±0·025	17·2±0·8	2·07±0·10
5	ATP+α-oxoglutarate	25·7±0·15	33·7±0·2	3·83±0·025	18·0±0·3	1·89±0·04
10	None	26·8±0·2	22·4±0·3	2·80±0·08	17·3±0·5	2·08±0·06
10	ATP+α-oxoglutarate	27·2±0·1	32·7±0·3	3·71±0·03	17·6±0·6	1·86±0·06
20	None	24·0±0·9	15·3±0·4	1·84±0·05	14·0±0·6	1·69±0·08
20	ATP+α-oxoglutarate	26·4±0·1	28·9±0·2	3·29±0·03	17·4±0·1	1·83±0·01
60	None	24·6±0·1	13·6±0·1	1·64±0·01	14·4±0·1	1·73±0·01
60	ATP+α-oxoglutarate	28·1±0·1	20·7±0·2	2·36±0·03	16·8±0·3	1·77±0·03

Table 3. *Effects of adenosine triphosphate and substrates on sodium, potassium and water contents of cortical and medullary rabbit-kidney mitochondria*

Conditions were similar to those described in Table 1, with the following exceptions. In Expt. 1, the final concentration of $MgSO_4$ was 2 mM instead of 5 mM. In all three experiments only sodium ATP was used. In Expt. 3, the mitochondria were not washed and incubations were for 15 min. In Expt. 3 b, 0·5 ml. of the mitochondrial suspension was added to 3·5 ml. of incubating medium in which the sodium and potassium phosphate buffers had been replaced by 10 mM-tris–HCl, pH 7·4. The final concentrations of ATP and L-glutamate in Expt. 3 were 1 and 20 mM respectively.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
Expt. 1					
Whole kidney, control	22·6, 22·6	12·3, 12·5	1·50, 1·52	14·8, 14·8	1·81, 1·81
Whole kidney, ATP+glutamate	28·4±0·1	21·0±0·1	2·57±0·02	19·9±0·7	1·97±0·07
Cortex, control	24·3±0·1	13·0±0·3	1·59±0·03	15·0±0·4	1·83±0·04
Cortex, ATP+glutamate	26·3±0·3	21·4±0·3	2·61±0·04	19·1±0·3	1·89±0·03
Medulla, control	18·3±0·7	12·9±0·4	1·57±0·06	20·1±0·7	2·45±0·09
Medulla, ATP+glutamate	21·5±0·2	14·1±0·3	1·72±0·04	23·7±1·6	2·27±0·15
Expt. 2					
Cortex, control	23·5±0·4	14·3±0·5	1·77±0·07	21·8±0·8	2·48±0·10
Cortex, ATP+α-oxoglutarate	24·9±0·4	27·7±0·2	3·42±0·03	25·1±1·0	2·34±0·09
Cortex, ATP+succinate	24·8±0·3	28·1±0·1	3·47±0·01	25·7±0·6	2·40±0·07
Medulla, control	17·1±0	13·4±0·2	1·66±0·03	25·9±1·0	2·94±0·12
Medulla, ATP+α-oxoglutarate	22·4, 20·2	18·8, 18·9	2·32, 2·34	33·0, 34·6	3·08, 3·23
Medulla, ATP+succinate	22·4, 21·8	19·1, 18·1	2·36, 2·24	34·7, 34·4	3·24, 3·21
Expt. 3					
Control (a)	19·4±0·1	13·5±0·3	1·18±0·02	14·4±0·1	1·13±0·01
ATP+glutamate (a)	21·6, 22·1	22·8, 23·2	2·00, 2·04	17·2, 17·8	1·14, 1·18
Control (b)	19·1±0·1	4·7±0·1	3·62±0·12	6·2±0·5	4·77±0·41
ATP+glutamate (b)	22·3±0·1	14·2±0·2	10·92±0·17	8·9±0·3	2·34±0·09

Comparison of sodium and potassium transport by cortical and medullary mitochondria. Table 3 presents experiments in which movement of the two ions was studied with mitochondria isolated from the whole kidney, the cortex or the medulla. In the first experiment, with the 8–10 medium, the potassium gradient was maintained at a higher value by ATP and glutamate in mitochondria from the whole kidney and the cortex, than from medullary mitochondria. The sodium gradients did not change with the addition of ATP and substrate.

Since the medullary mitochondria maintained the potassium gradient with the addition of ATP and glutamate only slightly better than the control, this experiment was repeated with 5 mM- instead of 2 mM-$MgSO_4$. In this experiment a marked decrease in the loss of potassium occurred, the gradient being maintained at 3·06 in the presence of ATP and glutamate, where the control value was 1·66. The reason for these differences is not clear. It might be due to a higher magnesium requirement for potassium retention by the medullary mitochondria.

The second experiment (Table 3), which was also performed with the 8–10 medium, shows that with α-oxoglutarate or succinate the results obtained were similar to those with glutamate. However, the maintenance of the potassium gradients was significantly less with the medullary mitochondria than with those from the cortex. Measurement of the oxygen uptake by medullary mitochondria with the conventional Warburg technique with air as the gas phase and with the same media used for the test of potassium retention, showed that the uptake of oxygen with glutamate, α-oxoglutarate and succinate respectively was 237, 301 and 375·5 μl. of air/2·5 ml. of suspension/½ hr., the control value being 6.

When both the 0–1 medium and the 8–10 medium were used with the same preparation of unwashed medullary mitochondria (Table 3, Expt. 3), addition of ATP and glutamate to the medium resulted in significantly greater potassium gradients' being maintained by the particles. In Expt. 3b (Table 3), although the sodium content of the mitochondria incubated with ATP and glutamate was greater than that of the control, the sodium gradient was less. This is probably the result of using the sodium salt of ATP in the incubating medium, the resulting increase in the sodium concentration of the medium being greater than the increased sodium concentration in the mitochondria.

In comparing the movements of sodium, potassium and water by medullary and cortical mitochondria, a number of differences were found. First, the potassium gradients maintained by washed cortical mitochondria with ATP and substrate usually were significantly greater than those maintained by the medullary mitochondria. Secondly, the water content of the medullary mitochondria was significantly greater than that of the cortical mitochondria. However, addition of ATP and substrate to the medium resulted in extrusion of water from both sorts of mitochondria. Thirdly, on a dry-weight basis, the amount of mitochondrial material isolated from the cortex was two to three times that from the medulla.

Experiments with the 0–1 medium. Table 4 shows that the loss of sodium from kidney mitochondria is different from that of potassium. In the first experiment, the mitochondria were suspended in a solution containing approx. 8 mM-sodium, and the suspension was added to a solution containing approximately the same amount of potassium but no sodium. ATP and α-oxoglutarate had virtually no effect on the sodium content, which decreased 26 % during 40 min. incubation. Loss of potassium was significantly decreased after 20 and 40 min. incubation by the addition of nucleotide and substrate.

In the second experiment, the mitochondrial suspension was added to a medium containing only the sodium and potassium contributed by the sodium and potassium salts of ATP. A greater potassium gradient was maintained when the nucleotide and α-oxoglutarate were added to the medium. Although the sodium content of the particles was somewhat greater with ATP and substrate, the sodium gradient was less than that of the control. This observation is similar to the one in Expt. 3b (Table 3).

The third experiment was designed primarily to determine whether a massive amount of ATP (25 mM) and substrate would cause an increase in the sodium gradient. It had been demonstrated that if arsenite was added to a suspension of heart mitochondria together with 1 mM-ATP and 20 mM-α-oxoglutarate, the usual increase in the potassium gradient was prevented (Ulrich, 1960*a*). This inhibition could be partially overcome by increasing the ATP concentration 25-fold. The third experiment shows that even with 25 mM-ATP, the sodium gradient after incubation did not differ from the incubated control. The potassium gradients, which reflect the loss of endogenous potassium from the mitochondria (no potassium having been present in either the suspending or incubating media), were greater with the addition of ATP and substrate, i.e. these two compounds caused decreased loss of the ion from the particles. In this third experiment, an amount of NaCl approximately equal to the sodium in the ATP was added to the control solutions.

Effects of pH. In these experiments, only the pH of the incubating media was varied, the pH of the

suspending medium, which in most of the experiments was 6·9–7·1, being kept constant. In the first experiment, with the 8–10 medium (Table 5), increasing the pH from 6·2 to 7·4 caused a significant decrease in the loss of potassium, the control potassium gradient increasing from 2·40 at pH 6·2 to 3·65 at pH 7·4. These changes did not occur when ATP and α-oxoglutarate were added to the incubating medium. In fact, the potassium gradients decreased slightly as the pH was increased. The loss of sodium from the mitochondria was virtually unchanged as the pH was altered.

The second experiment (8–10 medium) was designed to study changes in the loss of potassium over a wider pH range when the mitochondrial suspensions were centrifuged immediately after being added to the incubating media. The buffering range of the tris and phosphate buffers used is such that at the lowest pH values studied, the pH of the solutions increased from 4·5 to 5·6 and from 5·5 to 5·9 when 0·5 ml. of the suspending medium (not containing mitochondria) was added to 3·5 ml. of the incubating medium. Nevertheless, the results show that, as the pH of the incubating medium was raised, the loss of potassium was significantly decreased. Again there was virtually no change in the sodium content of the mitochondria.

The effects of pH on the potassium gradients are illustrated even more strikingly in the third experiment, in which the 0–1 medium was used. As the pH of the incubating medium was increased from 6·2 to 7·8, the control potassium gradient increased more than fourfold, from 4·92 to 20·70. Again, changes in pH had little, if any, effect on the potassium gradients when ATP and α-oxoglutarate were added to the incubating medium. The sodium gradient also was not affected.

That these effects of pH were not due to the pH of the incubating media, but probably to some sort of exchange of H^+ ion for K^+ ion, was shown by an experiment in which mitochondria were suspended

Table 4. *Effects of adenosine triphosphate and α-oxoglutarate on loss of sodium and potassium from rabbit-kidney mitochondria*

In Expt. 1, the mitochondria were suspended with 0·25 M-sucrose, 5 mM-$MgSO_4$ and 5 mM-NaH_2PO_4,H_2O–Na_2HPO_4 (pH 7·4). A portion (0·5 ml.) of the suspension was added to 3·5 ml. of incubating medium consisting of 0·25 M-sucrose, 5 mM-$MgSO_4$ and 5 mM-K_2HPO_4–KH_2PO_4 (pH 7·4), together with (or without) potassium ATP and α-oxoglutarate to give a final concentration of 1 mM and 20 mM respectively. In Expt. 2, the mitochondria were suspended with the same solution as in Expt. 1. However, 5 mM-K_2HPO_4–KH_2PO_4 (pH 7·4) had been added to the solution. A portion (0·5 ml.) of the suspension was added to 3·5 ml. of incubating medium which consisted of 0·25 M-sucrose, 5 mM-$MgSO_4$ and 10 mM-tris (pH 7·4) together with (or without) potassium ATP, sodium ATP and α-oxoglutarate to give a final concentration of 0·5 mM, 0·5 mM and 20 mM respectively. In Expt. 3, the mitochondria were suspended with 0·25 M-sucrose, 5 mM-$MgSO_4$ and 10 mM-Na_2HPO_4–NaH_2PO_4,H_2O (pH 7·4). A portion (2 ml.) of the suspension was added to 2 ml. of incubating medium with the same composition together with (or without) sodium ATP and α-oxoglutarate to give a final concentration of 25 and 20 mM respectively. Incubation times are shown in parentheses after the details of the experiments.

Expt. 1	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
Control (no incubation)	27·4, 26·9	26·7, 27·8	3·82, 3·92	9·6, 11·4	10·91, 12·96
ATP + α-oxoglutarate (no incubation)	27·5±0·1	33·6±0·5	3·86±0·04	11·8±0·7	11·89±0·72
Control (20 min.)	25·6±0·6	12·7±0·1	1·72±0·02	9·2±0·6	10·54±0·71
ATP + α-oxoglutarate (20 min.)	27·9±0·1	28·3±0·1	3·11±0·02	9·4±0·3	9·12±0·30
Control (40 min.)	24·7±0·6	11·8±0·1	1·56±0·01	8·0±0·8	8·66±0·86
ATP + α-oxoglutarate (40 min.)	27·4±0·4	22·8±0·3	2·48±0·02	8·3±0·5	8·17±0·54
Expt. 2					
Control (no incubation)	25·6±0·2	12·9±1·0	11·73±0·90	9·1±0·1	11·37±0·16
ATP + α-oxoglutarate (no incubation)	27·0±0·3	20·1±0	11·17±0	10·4±0·2	5·22±0·09
Control (20 min.)	21·6±0·1	3·7±0·1	2·64±0·04	6·3±0·1	7·00±0·08
ATP + α-oxoglutarate (20 min.)	24·9±0·1	10·4±0·2	4·95±0·09	8·5±0·3	4·25±0·14
Control (40 min.)	19·8±0	2·9±0	2·15±0	5·5±0·1	6·11±0·13
ATP + α-oxoglutarate (40 min.)	24·0±0·1	6·1±0·1	2·54±0·02	7·7±0·2	3·85±0·11
Expt. 3					
Control (no incubation)	27·8±1·03	26·6±0·5	120·9±2·5	84·1±2·8	1·18±0·04
ATP + α-oxoglutarate (no incubation)	27·5±0·2	26·9±0·3	149·3±1·8	76·8±3·0	1·10±0·04
Control (20 min.)	27·0±0·2	5·3±0·1	8·03±0·23	87·2±1·8	1·23±0·02
ATP + α-oxoglutarate (20 min.)	26·5±0·5	14·0±0·1	31·1±0·2	86·9±1·2	1·24±0·02
Control (40 min.)	26·4, 26·6	4·7, 4·5	6·7, 6·3	88·2, 90·4	1·27, 1·24
ATP + α-oxoglutarate (40 min.)	28·6, 28·1	10·9, 10·4	19·3, 19·8	80·6, 82·1	1·15, 1·18

Table 5. *Effects of* pH *on sodium and potassium contents of rabbit-kidney mitochondria*

In Expt. 1 the mitochondria were suspended with the same solution as in Table 1. A portion (0·5 ml.) of the suspension was added to 3·5 ml. of incubating medium of the same composition together with (or without) sodium ATP and α-oxoglutarate to give final concentrations of 1 mM and 20 mM respectively. The pH of the incubating media was adjusted with either solid tris (ATP and α-oxoglutarate) or solid tris or HCl (control) or both. Suspensions were incubated for 20 min. In Expt. 2, no ATP or α-oxoglutarate was added and the suspensions were centrifuged immediately after addition of the mitochondria to the incubating medium. Figures in parentheses refer to the pH resulting when 0·5 ml. of the solution used for suspending the mitochondria was added to 3·5 ml. of the incubating medium. In Expt. 3, the mitochondria were suspended with the same solution as in Expts. 1 and 2. A portion (0·5 ml.) of the suspension was added to 3·5 ml. of a solution consisting of 0·25 M-sucrose, 5 mM-$MgSO_4$ and 10 mM-tris–HCl buffer (pH 7·4). When added, sodium ATP, potassium ATP and α-oxoglutarate were present at final concentrations of 0·5, 0·5 and 20 mM respectively. Incubations were carried out for 15 min.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
Expt. 1					
pH 6·2, control	26·6±0·3	19·9±0·1	2·40±0·01	16·7±0·2	2·04±0·03
pH 6·2, ATP+α-oxoglutarate	26·5±0·1	34·2±0·2	4·12±0·03	17·1±0·8	1·68±0·07
pH 6·8, control	26·7±0·1	26·6±0·3	3·20±0·03	18·2±1·0	2·22±0·12
pH 6·8, ATP+α-oxoglutarate	26·9±0·2	34·3±0·5	4·13±0·05	17·7±0·8	1·74±0·08
pH 7·4, control	28·5±0·1	30·3±0·3	3·65±0·03	18·3±0·4	2·23±0·05
pH 7·4, ATP+α-oxoglutarate	29·4±0·2	32·0±0·3	3·86±0·04	17·5±0·2	1·71±0·04
pH 7·8, control	28·0±0·1	30·4±0·2	3·66±0·02	17·3±0·2	2·11±0·03
pH 7·8, ATP+α-oxoglutarate	28·1±0·1	29·9±0·2	3·60±0·03	16·8±0·1	1·65±0·01
Expt. 2					
pH 4·5 (5·6)	28·4±0·1	20·6±0·2	2·58±0·03	18·9±0·8	2·42±0·10
pH 5·5 (5·9)	27·7±0·2	20·6±1·1	2·58±0·14	19·1±1·6	2·45±0·21
pH 6·4 (6·5)	26·6±0·4	21·9±0·3	2·74±0·03	17·6±0·7	2·26±0·09
pH 6·9 (6·9)	25·4±0·1	23·7±0·4	2·96±0·06	18·0±0·3	2·31±0·03
pH 7·4 (7·4)	26·5±0·5	28·8±0	3·60±0	19·7±0·2	2·53±0·02
pH 8·4 (8·3)	27·1±0·2	32·1±0·1	4·01±0·01	19·1±0·1	2·45±0·01
Expt. 3					
pH 6·2, control	26·5±0·9	6·4±0·3	4·92±0·23	12·8±0·6	14·22±0·65
pH 6·2, ATP+α-oxoglutarate	27·5±0·2	26·7±0·3	14·83±0·15	14·9±0·2	7·84±0·09
pH 6·8, control	26·5±0·1	7·1±0·3	5·46±0·23	14·5±0·9	16·11±0·96
pH 6·8, ATP+α-oxoglutarate	28·4±0	27·5±0·2	15·28±0·11	15·1±0·9	7·95±0·45
pH 7·4, control	28·6±0·3	11·8±0·3	9·83±0·22	12·1±0·7	13·45±0·76
pH 7·4, ATP+α-oxoglutarate	29·0±0·2	26·9±0·2	14·94±0·09	13·7±0·2	7·21±0·09
pH 7·8, control	29·2±0·3	20·7±0·5	20·70±0·47	12·6±0·5	14·00±0·59
pH 7·8, ATP+α-oxoglutarate	28·8, 28·4	24·4, 24·3	13·55, 13·50	13·3, 12·6	7·00, 6·64

in media at pH 6·2, 6·8 or 7·4 and added to incubating media with the same pH as the suspending media. Under these conditions, increasing the pH from 6·2 to 7·4 had no effect on the loss of potassium from the particles.

Effects of arsenite and 2:4-*dinitrophenol on potassium gradients.* It had been demonstrated (Ulrich, 1960*a*) that a variety of metabolic poisons, including those which uncouple oxidative phosphorylation, inhibited an increase in the potassium gradient maintained by rabbit-heart mitochondria. In the present study with the 0–1 medium containing AMP and α-oxoglutarate, addition of relatively large concentrations of arsenite and 2:4-dinitrophenol caused an increased loss of potassium from the mitochondria (Table 6, Expt. 1). In the second experiment, carried out with twice the concentration of potassium and no magnesium in the medium, the effects of 2:4-dinitrophenol were more striking. At concentrations of 1 or 0·1 mM, the loss of potassium was greater than in the first experiment. The effect of 1 mM-2:4-dinitrophenol was similar whether AMP or ATP was used.

Other experiments designed to demonstrate an increase in the sodium gradient. A number of other experiments were performed in an attempt to demonstrate increased sodium gradients in the presence of ATP and substrate. These experiments were carried out with the 8–10 medium. Thus 2 mM-pyridoxal, alone or with ATP, was without effect. Incubating the mitochondria in the usual media but with 0·125 M-, 0·25 M- or 0·50 M-sucrose also had no effect. Finally, an experiment was performed to rule out the possible effect of changes in ion gradients during the centrifuging that follows incubation of the mitochondria. The suspensions

Table 6. *Effects of 2:4-dinitrophenol and arsenite on loss of potassium from rabbit-kidney mitochondria*

In Expt. 1 the mitochondria were suspended with the same solution as in Table 1. A portion (0·5 ml.) of the mitochondrial suspension was added to 3·5 ml. of 0·25M-sucrose, 5 mM-$MgSO_4$ and 10 mM-tris–HCl (pH 7·4) together with sodium AMP and α-oxoglutarate to give a final concentration of 1 mM and 20 mM respectively. In Expt. 2 the mitochondria were suspended with 0·25M-sucrose and 10 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), and 0·5 ml. of the suspension was added to 3·5 ml. of 0·25M-sucrose and 10 mM-tris–HCl (pH 7·4). The final concentration of sodium ATP was 1 mM. In both experiments, the mitochondria were incubated for 15 min. DNP, 2:4 Dinitrophenol.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents $\left(\frac{\text{mitochondria}}{\text{medium}}\right)$
Expt. 1			
Control	23·8±0·3	7·5±0·1	6·25±0·05
AMP+α-oxoglutarate	26·2±0·3	21·4±0·3	17·84±0·21
AMP+α-oxoglutarate+1 mM-arsenite	25·3±0·2	7·2±0·3	6·00±0·27
AMP+α-oxoglutarate+1 mM-DNP	26·9, 28·5	16·0, 17·3	13·33, 14·42
AMP+α-oxoglutarate+0·01 mM-arsenite	29·0±0·3	20·4±0·3	17·00±0·27
AMP+α-oxoglutarate+0·01 mM-DNP	28·9±0·1	18·8±0·3	15·66±0·28
Expt. 2			
Control	21·6±0	6·2±0·1	2·14±0·03
AMP+α-oxoglutarate	26·9±0	21·4±0·6	7·38±0·19
AMP+α-oxoglutarate+1 mM-DNP	26·4±0·3	9·3±0·3	3·21±0·10
AMP+α-oxoglutarate+0·1 mM-DNP	25·2±0·6	10·0±0·1	3·45±0·03
AMP+α-oxoglutarate+0·01 mM-DNP	26·8, 26·8	16·9, 16·6	5·83, 5·72
ATP+α-oxoglutarate+1 mM-DNP	25·8±0·3	9·9±0·2	3·41±0·05

were centrifuged for either the standard 5 min. or for 1 or 2 min. After the 5 min. centrifuging, half the tubes were allowed to stand for 10 min. before the supernatant was poured off. The only significant change in this experiment was that the percentage dry weight of the mitochondria isolated after 1 or 2 min. centrifuging (25·6 %) was less than that of the 5 min. centrifuging (27·9 %). ATP and α-oxoglutarate did not increase the sodium gradient. A slight decrease of the sodium gradient occurred with these additions (from 1·87 to 1·55), but these changes were so small that their significance is doubtful.

DISCUSSION

Possible reasons for inability to demonstrate increased sodium gradients. One of the main objects of the present study was to determine whether the sodium gradients developed by kidney mitochondria during incubation at 37° could be increased in the presence of an oxidizable substrate and ATP. In other words, whether metabolic activity could decrease the loss of sodium from the particles. Since the evidence presented in this paper is completely negative on this point, it is necessary to consider whether this might be due to the conditions of the experiments.

The sodium and potassium concentrations in the mitochondria were calculated from the water content of the whole mitochondrial pellet. This would include intermitochondrial (extraparticulate) water which Werkheiser & Bartley (1957) and Jackson & Pace (1956) estimated to account for 20 % or less of the total water of liver-mitochondrial pellets. Amoore & Bartley (1958) reported variations as great as 24–59 % in the extraparticulate water of liver mitochondria from different preparations. It is possible therefore that changes in intramitochondrial sodium could be obscured by extraparticulate sodium, especially if the extraparticulate water content were as great as 59 %. Although the response was not uniform, in most of the experiments addition of ATP and substrate to the mitochondrial suspension resulted in extrusion of water from the particles. If sodium were not extruded with water, its concentration in mitochondrial water could have risen without a marked change in the sodium content of the pellet. However, it is doubtful whether the small changes in mitochondrial water would have obscured marked changes in mitochondrial sodium.

Although these two criticisms cannot be answered satisfactorily, the decreased loss of potassium in the presence of ATP and substrate was of such magnitude that small variations in the extraparticulate concentration of this ion could not have caused the observed changes. Since these experiments were designed to measure only changes in relative ion concentration, the contribution of the extraparticulate water can probably be disregarded if one assumes that it is constant in each mitochondrial preparation (cf. Jackson & Pace, 1956).

The inability to demonstrate increased sodium gradients might have been due to loss of these gradients between the time that incubation was

stopped and the supernatant removed after centrifuging of the suspension. Also, since the mitochondria were not washed after incubation and centrifuging, an increased sodium gradient might have been obscured if the increase were limited to a particular sodium fraction.

Mitochondrial sodium studies by other investigators. A number of investigators have reported that the sodium and potassium gradients developed by sheep-kidney mitochondria (Bartley & Davies, 1954) and rat-liver mitochondria (Macfarlane & Spencer, 1953; Price, Fonnesu & Davies, 1956) could be increased by incubation in the presence of an oxidizable substrate and an adenine nucleotide. The experimental conditions of the present study, although not identical, were similar to those of these three groups. Their data for sodium was so variable that the results must be viewed with caution. With kidney mitochondria, Bartley & Davies (1954) usually were unable to demonstrate concentration gradients greater than 1·0 for sodium or potassium when they studied the distribution of the radioactive ions between the particles and medium. In the same paper (Table 7), Bartley & Davies presented data not only for the development of both sodium and potassium gradients but also for significant increases in these gradients in the presence of α-oxoglutarate and ATP.

Macfarlane & Spencer (1953) observed that the sodium and potassium contents of mitochondria incubated for 10 min. in a medium containing AMP and substrate were low, but doubled in the next 20 min. incubation. This could not be confirmed by Price *et al.* (1956), who carried out a detailed study of ion and water movements by rat-liver mitochondria. Although Price and co-workers were able to demonstrate consistently increased potassium gradients when ATP or AMP was added to a medium containing oxidizable substrate, their results for increased sodium gradients were not consistent. Price and co-workers also studied the effects of varying the concentrations of phosphate buffer and Mg^{2+} ion (their Table 6). They found that increasing the phosphate concentration 10- or 30-fold had no effect on sodium transport. However, decreasing the Mg^{2+} ion concentration from 20 to 2 mM resulted in increased sodium gradients when AMP was added to the medium. In the present series of experiments, decreasing the magnesium concentration from 5 to 2 mM had no effect on sodium transport (compare Expts. 1 and 2 in Table 1 with Expt. 1 in Table 3).

Perfusion of the kidneys *in situ* with iso-osmotic salt solution and sucrose caused the disappearance of a small amount of dark-red pigment at the bottom of the mitochondrial pellet, but had no effect on the loss of sodium and potassium when compared with isolated mitochondria from unperfused kidneys.

It is possible that the differences between the results in this paper and those of the three groups mentioned above are due to species differences. However, both Spector (1953) and Jackson & Pace (1956) found that the sodium gradients maintained by rat-liver mitochondria could not be increased.

Potassium transport. The greater potassium gradients maintained by rabbit-kidney mitochondria in the presence of an adenine nucleotide and oxidizable substrate confirms previous observations on mitochondria from sheep kidney (Bartley & Davies, 1954), rat liver (Macfarlane & Spencer, 1953; Spector, 1953; Price *et al.* 1956), rat heart and skeletal muscle (Ulrich, 1959) and rabbit heart (Ulrich, 1960*a*). Does this mean that potassium is actively transported by mitochondria? In a study with isolated heart mitochondria in which a variety of metabolic inhibitors were added to the incubating media, some evidence was presented for possible active transport (Ulrich, 1960*a*). However, lack of knowledge of the potential difference across the mitochondrial membrane makes it difficult to prove this point conclusively. A discussion of this question is given by Amoore (1960). He found a lower concentration of chloride within the mitochondria than in the medium, which suggested that there might be a potential difference of the correct sign.

One series of observations in this study which is difficult to explain are the effects of pH on the potassium gradients. The increased gradients resulting from increasing the pH of the incubating medium might be due to a competition of H^+ and K^+ ions for a common site on a membrane complex or carrier. Since the gradients appeared to have reached a maximum when ATP and substrate were added to the incubating medium, it is not surprising that under these conditions changes in pH appeared to have no effect.

The sodium gradients maintained by mitochondria were not affected by changes in pH. Two observations may have a bearing on this finding. First, when heart or skeletal-muscle mitochondria are aged, the potassium content decreases approximately by one-half whereas the sodium content remains the same (Ulrich, 1959). Secondly, the specific activity of sodium (μg. of sodium/mg. dry wt.) was found to be several times as great in mitochondrial membrane fractions as in intact particles, whereas the reverse was found with potassium (Ulrich, 1960*b*). One interpretation of the lack of effect of pH is that mitochondrial sodium is located in the membrane, possibly firmly bound, and cannot compete with H^+ ions for some common site on the membrane.

SUMMARY

1. The loss of sodium and potassium from isolated rabbit-kidney mitochondria was studied. Addition of ATP and an oxidizable substrate to the incubating medium at 37° caused a significant decrease in the loss of potassium from the mitochondria, but had no effect on the sodium content of the particles.

2. Increasing the pH of incubating medium not containing adenosine triphosphate and substrate by small increments from 6·2 to 7·8 resulted in a significant decrease in the loss of potassium from the mitochondria. These changes in potassium were not observed when adenosine triphosphate and substrate were added to the medium. The sodium content of the particles was not altered by changes in pH.

3. Mitochondria isolated from either the cortex or the medulla of the kidney treated sodium and potassium in a manner similar to those from the whole kidney. However, adenosine triphosphate and substrate did not appear to inhibit loss of potassium from medullary mitochondria as effectively as from cortical mitochondria.

4. Addition of arsenite or 2:4-dinitrophenol to a medium containing adenosine monophosphate and substrate caused a marked increase in the loss of potassium.

5. Possible explanations are presented for the inability to demonstrate decreased loss of sodium in the presence of an oxidizable substrate and adenine nucleotide, and some conflicting results of sodium movements in liver and kidney mitochondria by other investigators are discussed.

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Acetoacetate as Fuel of Respiration in the Perfused Rat Heart

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Kulka, Krebs & Eggleston (1961) found that sheep-heart homogenates readily oxidize added acetoacetate. The ratio (oxygen used/acetoacetate oxidized) reached values of up to 4, indicating that acetoacetate was the sole or main fuel of respiration. It suppressed the oxidation of endogenous substrates.

Experiments on the isolated perfused rat heart are reported in this paper which show that the intact heart of the rat likewise uses acetoacetate as a preferred substrate of oxidation, even in the presence of glucose and insulin.

EXPERIMENTAL

Perfusion method. The perfusion apparatus was similar to that described by Bleehen & Fisher (1954) except that the anti-frothing device was omitted as frothing presented no difficulty. The gas mixture of O_2+CO_2 (95:5, v/v) was saturated with water vapour at 37° before being passed to the gas lifts, in order to avoid loss of water from the perfusion fluid by evaporation. The perfusate volume was usually between 17 and 25 ml. The bath temperature was 37°.

Male rats (Wistar strain) weighing between 220 and 280 g. were used. 'Well-fed' animals had unrestricted

access to food and water. 'Unfed' rats were without food for 24 hr. After the rats were anaesthetized with ether, the heart was rapidly removed and placed in a dish of cold (4°) saline medium (Krebs & Henseleit, 1932). The aorta was stripped of connective tissue and tied on to a cannula through which dripped perfusion medium at room temperature. At this temperature the heart began to beat again, though at a slow rate. A few beats expelled the blood from the coronary vessels, and the cannulated heart was then transferred to the perfusion apparatus. The time between the opening of the thorax and the start of the perfusion was usually less than 2 min.

According to Fisher & Williamson (1961) the O_2 consumption and the coronary flow of the perfused heart preparation falls by 10–20% within the first 15 min. of perfusion and then remains fairly constant for periods of up to 2 hr. In most of the present experiments a period of 15 min. was therefore allowed to elapse before the rate measurements of the metabolic changes began. During this 15 min. period the perfusate equilibrated with the tissue. In most experiments the zero samples (usually 1 ml.) were removed after 15 min. The perfusion was continued for 1 hr., the final samples being collected 75 min. after the start.

Whereas the changes in the perfusate of acetoacetate, β-hydroxybutyrate, glucose and lactate and also the final glycogen content of the tissue could be determined on one and the same heart, the O_2 consumption during perfusion and the glycogen content of the heart after perfusion for 15 min. (zero time) had to be measured on hearts of other animals. Details of the standard error of the means and of the number of experiments are therefore given.

Reagents. Insulin (crystalline insulin containing 23·9 units/mg.) was dissolved in 0·94% NaCl containing 0·01N-HCl to give a concentration of 1 i.u./ml. This stock solution was stored as small samples in the deep-freeze. The final insulin concentration in the perfusion fluid was 2 milliunits/ml. (2 mU/ml.). A molar stock solution of acetoacetate was prepared according to Krebs & Eggleston (1945) and standardized by the method of Edson (1935). It was stored in the deep-freeze.

Analytical methods. The oxygen consumption was measured polarographically according to R. B. Fisher & J. R. Williamson (unpublished work). Acetoacetate was determined according to Walker (1954) in 0·5 ml. of the 50-fold-diluted perfusate. β-Hydroxybutyrate was estimated in 1 ml. of a fivefold-diluted perfusate according to Greenberg & Lester (1944) and Bessman & Anderson (1957). Acetoacetate was first removed by vigorous boiling of the solution in a test tube (25 mm. × 150 mm.) for 15 min. after the addition of 2 drops of conc. H_2SO_4, and water was added to replace the loss. Glucose when present was removed by copper–lime treatment. The values obtained by the two methods agreed well; the method of Bessman & Anderson was used in most experiments because it is quicker. In a few experiments β-hydroxybutyrate and acetoacetate were determined by an enzymic method based on the use of a soluble D(−)-β-hydroxybutyric dehydrogenase obtained from *Rhodopseudomonas spheroides* (D. H. Williamson & J. H. Mellanby, personal communication). Lactate was determined in 1 ml. of diluted medium by the method of Lehmann (1938) as modified by Wieland (1957), S. R. Elsden (personal communication) and H. A. Krebs & T. Gascoyne (unpublished work). Purified lactic-dehydrogenase preparation (Boeri, Cutole, Luzzati & Tosi, 1955) was used, and ferricyanide replaced by 2:6-dichlorophenol-indophenol, which increased the sensitivity. The perfusate was diluted to contain up to 0·2μmole of lactate/ml. Glucose was determined with glucose oxidase according to Huggett & Nixon (1957); 1 ml. of a 50-fold dilution of perfusate was used. Citrate was determined according to Taylor (1953), succinate according to Rodgers (1961), malate according to Hohorst, Kreutz & Bücher (1959) and α-oxoglutarate according to Holzer & Holldorf (1957).

For the determination of glycogen in the tissue, the heart was removed from the cannula, cut into two pieces and blotted, and each piece was weighed on a torsion balance. One was used for the determination of the dry weight and the other was dropped into a centrifuge tube, containing 2 ml. of 30% KOH, placed in a boiling-water bath. The glycogen was separated by the method of Good, Kramer & Somogyi (1933) and hydrolysed by adding 3 ml. of 3N-H_2SO_4 and heating in a boiling-water bath for 2·5 hr. The hydrolysate was neutralized with NaOH and made up to a standard volume, and the amount of glucose in the solution was estimated by the glucose-oxidase method.

RESULTS

Disappearance of acetoacetate and formation of β-hydroxybutyrate. As shown in Table 1, acetoacetate at an initial concentration of 5 mM disappeared rapidly from the perfusion medium. The rate remained constant over the first 45 min. by which time the acetoacetate concentration had fallen to about 2·5 mM. The disappearance of acetoacetate was accompanied by the formation of β-hydroxybutyrate, the amounts being about half of the acetoacetate removed. As no appreciable quantities of the intermediates of the tricarboxylic acid

Table 1. *Rate of disappearance of acetoacetate and formation of β-hydroxybutyrate in the perfusion medium*

Heart (121 mg. dry wt.) from well-fed rat was initially perfused with 25 ml. of medium. Samples (1 ml.) were removed for analysis at the times stated.

Time (min.)	0	15	30	45	60	75
Concn. of acetoacetate (mM)	4·80	4·00	3·30	2·55	1·90	1·43
Concn. of β-hydroxybutyrate (mM)	0	0·38	0·71	0·96	1·23	1·28
Acetoacetate removed (μmoles)	—	20·0	36·8	54·0	68·3	78·2
β-Hydroxybutyrate formed (μmoles)	—	9·5	17·4	23·2	29·1	30·2
Acetoacetate 'oxidized' (μmoles)	—	10·5	19·4	30·8	39·2	48·0
Rate of acetoacetate oxidation for consecutive 15 min. periods (μmoles/g. dry wt./hr.)	-	48	296	377	278	292

cycle appeared in the medium or in the tissue (see below), the acetoacetate not accounted for by β-hydroxybutyrate was taken to be completely oxidized, and is therefore referred to as acetoacetate 'oxidized'. The rate of acetoacetate oxidation calculated in this way remained constant over the whole 75 min. period of perfusion (Table 1), although the rate of acetoacetate removal decreased after 45 min., this decrease being paralleled by a decreased β-hydroxybutyrate formation. That the fall of acetoacetate removal was not due to a deterioration of the metabolic capacity of the heart but to a decrease in the acetoacetate concentration was shown by the fact that, in experiments with a larger volume of perfusion fluid, the rate of acetoacetate removal and β-hydroxybutyrate formation remained constant over the whole 75 min. period of perfusion.

Another experiment in which the acetoacetate concentration was nearer the physiological value (initial concentration 0·25 mM) is shown in Table 2. Acetoacetate was still rapidly removed until the concentration fell to about one-tenth of the starting value. Hardly any β-hydroxybutyrate was formed. Whereas the rates of acetoacetate oxidation were not affected by the concentration between 2 and 5 mM, they fell gradually with the concentration between 0·1 and 1 mM. This follows from the values given for the rates of acetoacetate oxidation in Tables 1 and 2.

Oxidation of D(−)-*β-hydroxybutyrate.* An experiment in which D(−)-β-hydroxybutyrate was the substrate added to the perfusate is shown in Table 3. The medium containing D(−)-β-hydroxybutyrate was prepared by perfusing hearts for 75 min. with 25 ml. of medium containing 10 mM-acetoacetate. The perfusate was placed in a boiling-water bath for 60 min. to decompose most of the remaining acetoacetate, and a stream of N_2 was bubbled through the solution to remove the acetone. The $CaCO_3$ formed during this treatment was redissolved after cooling by gassing with N_2+CO_2

Table 2. *Rate of disappearance of acetoacetate and of formation of β-hydroxybutyrate in the perfusion medium at low concentrations of acetoacetate*

Heart (141 mg. dry wt.) from well-fed rat was perfused initially with 50 ml. of medium containing 0·25 mM-acetoacetate. Samples (2 or 4 ml.) were removed for analysis at times stated. The values given in the last horizontal line are $100 \times a/n$ (see Discussion).

Time (min.)	0	10	20	30	40	50	60	70
Concn. of acetoacetate (mM)	0·250	0·175	0·125	0·090	0·065	0·044	0·019	0·012
Concn. of β-hydroxybutyrate (mM)	0	0·004	0·009	0·010	0·013	0·013	0·015	0·013
Acetoacetate removed (μmoles)	—	3·60	5·90	7·37	8·32	9·03	9·78	9·96
β-Hydroxybutyrate formed (μmole)	—	0·19	0·42	0·47	0·58	0·58	0·64	0·59
Acetoacetate 'oxidized' (μmoles)	—	3·41	5·48	6·90	7·74	8·45	9·14	9·37
Rate of acetoacetate oxidation for consecutive 10 min. periods (μmoles/g. dry wt./hr.)	—	145	88	60	35	30	29	10
Contribution of acetoacetate to substrate of respiration (%)	—	36	22	15	9	8	7	3

Table 3. *Rate of disappearance of* D-*β-hydroxybutyrate by the perfused rat heart*

Heart (126 mg. dry wt.) from well-fed rat was initially perfused with 25 ml. of medium containing 2 mM-D-β-hydroxybutyrate. Samples (1 ml.) were removed for analysis at the times stated. The values given in the last horizontal line are $100 \times a/n$ (see Discussion).

Time (min.)	0	15	30	45	60	75	90	105	120
Concn. of β-hydroxybutyrate (mM)	2·00	1·57	1·28	1·03	0·86	0·72	0·61	0·51	0·39
Concn. of acetoacetate (mM)	0·053	0·140	0·133	0·110	0·103	0·085	0·073	0·060	0·043
β-Hydroxybutyrate removed (μmoles)	—	10·3	17·0	22·5	26·1	28·9	31·0	32·8	34·8
Acetoacetate formed (μmoles)	—	2·09	1·93	1·42	1·27	0·91	0·68	0·45	0·16
β-Hydroxybutyrate 'oxidized' (μmoles)	—	8·23	15·1	21·1	24·8	28·0	30·3	32·3	34·6
Rate of β-hydroxybutyrate 'oxidation' over consecutive 15 min. periods (μmoles/g. dry wt./hr.)	—	261	217	191	118	100	74	64	74
Contribution of β-hydroxybutyrate to substrate of respiration (%)	—	73	61	54	33	28	21	18	21

(95:5, v/v). The concentration of β-hydroxybutyrate in the medium was 2·00 mM and that of acetoacetate 0·053 mM.

On perfusion β-hydroxybutyrate disappeared rapidly at the beginning and more slowly later (Table 3). Some acetoacetate was formed in the first 15 min. but this was subsequently utilized by the heart. The rates of β-hydroxybutyrate oxidation over consecutive 15 min. periods, calculated as the difference between the disappearance of β-hydroxybutyrate and formation of acetoacetate, are shown in the last-but-one horizontal line of Table 3. The rates were comparable with those of acetoacetate oxidation at similar concentrations (Table 2).

Accumulation of intermediates. In order to test whether the oxidation of acetoacetate was complete, the concentrations of the acids of the tricarboxylic acid cycle were measured in the heart tissue and in the perfusate. After 75 min. when another heart had oxidized 48 μmoles of acetoacetate (Table 1) less than 0·1 μmole, i.e. no significant amounts, of α-oxoglutarate, succinate and malate appeared in the perfusate, nor was there an appreciable change in the tissue concentrations of these intermediates (less than 0·1 μmole). On the other hand, 2·2 μmoles of citrate were found in the perfusate at 75 min. About 10% of this could be accounted for by a decrease of the tissue content of citrate. The origin of the citrate in the perfusate was not further investigated. A possible source of the oxaloacetate required for the synthesis of citrate is glutamate, which, according to Hicks & Kerly (1960), disappears from the tissue during perfusion under similar conditions at the rate of 1·2 μmoles in 60 min. Another possible source is CO_2 fixation by pyruvate, catalysed by the malic enzyme.

The findings indicate that by far the greater part of the acetoacetate not accounted for as β-hydroxybutyrate underwent complete oxidation.

Effect of glucose and insulin on acetoacetate metabolism. Glucose alone had no effect on the fate of acetoacetate, whereas insulin (without glucose) decreased the removal of acetoacetate and the formation of β-hydroxybutyrate in well-fed rats (Table 4). The insulin effects were independent of the initial acetoacetate concentration. Insulin decreased the oxidation of acetoacetate by 22–35% under all conditions tested, i.e. with or without glucose, and in unfed or well-fed animals. This effect was statistically significant (P 0·01). In unfed animals the inhibition of acetoacetate removal by insulin was not significant, but a larger proportion of the acetoacetate was reduced to β-hydroxybutyrate so that the amount of acetoacetate oxidized was still significantly decreased.

Table 4 also shows that the mean rate of acetoacetate removal was slightly greater at an initial concentration of 10 mM; but the rate of reduction to β-hydroxybutyrate increased at the same time so that the amounts of acetoacetate oxidized were not significantly affected by the acetoacetate concentration. There was no appreciable difference in the ketone-body metabolism of hearts from well-fed and unfed animals (Table 4).

Effect of acetoacetate on glucose metabolism. According to Bleehen & Fisher (1954), the perfused rat heart removes only small amounts of glucose from the perfused medium unless insulin is added. With insulin the rate of glucose removal becomes very rapid, and some of the glucose removed is recovered as tissue glycogen (Fisher & Lindsay, 1956). As shown in Table 5, insulin also causes a substantial increase in the formation of lactate, and the amount of glucose not accounted for by either glycogen or lactate, and presumably oxidized, was also increased.

In the absence of insulin acetoacetate caused no change in the rate of glucose uptake, but acetoacetate affected the fate of glucose in that less

Table 4. *Removal of acetoacetate and formation of β-hydroxybutyrate by the perfused rat heart: effects of glucose and insulin*

The values shown are means ± S.E.M. and, in parentheses, the numbers of experiments.

	Additions to perfusion medium (initial concn.)			Metabolic changes (μmoles/g. dry wt./hr.)		
Nutritional state	Acetoacetate (mM)	Glucose (mM)	Insulin (mU/ml.)	Acetoacetate	β-Hydroxybutyrate	Acetoacetate 'oxidized'
Well-fed	5	—	—	503±14 (8)	211±17 (8)	292±11 (8)
	5	—	2	377±18 (6)	172±21 (6)	205±15 (6)
Well-fed	10	—	—	566±12 (4)	224±11 (4)	322±6 (4)
	10	—	2	481±31 (6)	229±27 (6)	252±10 (6)
Unfed for 24 hr.	5	—	—	499±24 (10)	187±16 (10)	312±21 (10)
	5	—	2	461±23 (4)	257±23 (4)	204±22 (4)
Well-fed	5	5	—	515±5 (4)	205±11 (4)	310±12 (4)
	5	5	2	361±15 (7)	147±6 (7)	214±11 (7)

Table 5. *Effects of acetoacetate and insulin on glucose removal, lactate formation and glycogen synthesis in the perfused rat heart*

Glucose, lactate and the 75 min. value for glycogen were measured in one heart preparation simultaneously. Consumption of O_2 and the glycogen value for 15 min. were obtained from other preparations. Well-fed animals were used. The values shown are means ±S.E.M. and, in parentheses, the numbers of experiments. Glycogen is expressed as glucose equivalents.

Additions to medium (initial concn.)			Metabolic changes (μmoles/g. dry wt./hr.)							
					Glycogen					
Glucose (mM)	Acetoacetate (mM)	Insulin (mU/ml.)	Glucose	Lactate	Found at 15 min.	Found at 75 min.	Diff.	Glucose not accounted for by glycogen or lactate	O_2 uptake	Acetoacetate 'oxidized'
5	—	—	72±8 (5)	0 (5)	77±6 (4)	72±5 (5)	0	72	1680±27 (23)	—
5	—	2	266±7 (6)	114±14 (6)	95±6 (5)	140±9 (6)	+45	164	1734±30 (18)	—
5	5	—	71±13 (9)	83±22 (4)	78±10 (4)	96±5 (10)	+18	12	1591 (3)	310±12 (4)
5	5	2	129±16 (13)	95±21 (3)	108±11 (4)	146±14 (10)	+38	43	1637 (2)	214±11 (7)

Table 6. *Summary of data on oxygen consumption and substrate changes (acetoacetate, β-hydroxybutyrate, glucose, glycogen) in the perfused rat heart (contribution of acetoacetate and glucose to fuel of respiration)*

The data are compiled from Tables 4 and 5 and from R. B. Fisher & J. R. Williamson (unpublished work).

	Nutritional state of animal										
	Well fed	Well fed	Well fed	Well fed	Unfed	Unfed	Well fed	Well fed	Well fed	Well fed	Well fed
Additions to perfusion medium											
Acetoacetate (mM)	5	5	10	10	5	5	5	5	—	—	—
Glucose (mM)	—	—	—	—	—	—	5	5	5	5	—
Insulin (mU/ml.)	—	2	—	2	—	2	—	2	—	2	—
Acetoacetate unaccounted for as β-hydroxybutyrate (μmoles/g. dry wt./hr.)	292	205	322	252	312	204	310	214	—	—	—
Glucose unaccounted for as glycogen or lactate (μmoles/g. dry wt./hr.)	—	—	—	—	—	—	12	43	72	164	—
O_2 uptake (μmoles/g. dry wt./hr.)	1455						1607		1704		1700
Glycogen change (μmoles/g. dry wt./hr.)	+5	−16	−18	−54	−47	−29	+18	+38	−5	+45	−78
β-Hydroxybutyrate formed (μmoles/g. dry wt./hr.)	211	172	244	229	187	257	205	147	—	—	—
O_2 equivalents (μmoles/g. dry wt./hr.)	1561	1541	1577	1570	1549	1583	1710	1681	1704	1704	1700
$\frac{O_2 \text{ equivalents}}{\text{Acetoacetate oxidized}}$ (n)	5·33	7·54	4·90	6·22	4·96	7·75	5·52	7·86	—	—	—
$\frac{O_2 \text{ equivalents}}{\text{Glucose oxidized}}$ (n)	—	—	—	—	—	—	70	26	23	10	—
Contribution of acetoacetate as fuel (%)	75	53	82	64	81	52	73	51	—	—	—
Contribution of glucose or glycogen as fuel (%)	0	6	7	20	29	12	4	15	24	58	27

glucose underwent oxidation; instead almost all the glucose removed was converted into glycogen or lactate. In the presence of insulin, acetoacetate halved the glucose uptake, this decrease being almost entirely due to a suppression of glucose oxidation. The amounts of lactate and glycogen formed from glucose were not affected by acetoacetate.

Oxygen consumption. Data on the O_2 consumption are included in Table 5. Those for the medium without acetoacetate are taken from R. B. Fisher & J. R. Williamson (unpublished work). Values obtained in the presence of acetoacetate and glucose were not significantly different from those obtained in the presence of glucose alone. When acetoacetate was the sole added substrate slightly lower values were obtained [$1455 \pm 44(18)$ μmoles/g. dry wt./hr.]. Insulin or deprivation of food had no effect.

DISCUSSION

Acetoacetate and glucose as fuels of respiration in rat heart. The mean values of substrate disappearance based on Tables 4 and 5 and additional data, together with the oxygen consumption in the presence of the substrates, are summarized in Table 6. When acetoacetate was added it served as a hydrogen acceptor in addition to oxygen, and the total oxidative capacity was therefore the sum of oxygen consumption and β-hydroxybutyrate formation. This sum is expressed in Table 6 as 'O_2 equivalents'. The contribution made by β-hydroxybutyrate formation to the sum was about 5–8%. If the observed ratio (O_2 equivalents/substrate oxidized) is n and the O_2 equivalent required for complete oxidation of this substrate is a, the value $100 \times a/n$ expresses, as a per cent, the contribution that the substrate makes to the total respiration, provided that the oxidation is complete and does not lead to accumulation of intermediates. For acetoacetate a is 4, and for glucose a is 6.

The last-but-one vertical column of Table 6 shows that in the absence of insulin acetoacetate contributed 73–82% to the respiration. This was independent of the nutritional state of the rat or the presence of glucose. Insulin decreased the contribution made by acetoacetate to 51–64% and this decrease was independent of the presence of glucose. In other words, glucose did not suppress the oxidation of acetoacetate, even in the presence of insulin, but acetoacetate suppressed the oxidation of glucose; in the presence of acetoacetate most of the glucose removed was recovered either as glycogen or as lactate.

When glucose was the sole added substrate it contributed no more than 24% to the respiration. Insulin raised the contribution to 57%.

Under the test conditions three kinds of substances provided the fuel of respiration: (1) added acetoacetate, (2) added glucose, (3) endogenous substrates. As no ammonia, urea, glutamate or aspartate was formed (unpublished data; see also Hicks & Kerly, 1960), amino acids may be excluded as endogenous substrates. These substrates were presumably carbohydrate and fat. In the hearts from well-fed animals glycogen contributed at most 20% to the fuel of respiration when acetoacetate was present. Without insulin the contribution was even smaller (Table 6). Thus acetoacetate, when available, was the preferred fuel, though it did not completely suppress the use of endogenous substrates. Insulin shifted the proportion of acetoacetate to endogenous fuel in favour of the latter, raising it from 18–27% to 36–49%. The endogenous substrates were next to acetoacetate in the order of preference as fuel. The fact that the oxygen consumption of the heart was not raised by the addition of substrates indicates that endogenous material can maintain the normal rate of respiration for considerable periods. Glucose without insulin did not compete effectively with endogenous substrates, but when insulin was also added glucose contributed about half the fuel of respiration.

The values for the ratio (O_2 equivalents/acetoacetate oxidized) were of the same order as those obtained on sheep-heart homogenates (Kulka *et al.* 1961), as were the ratios (acetoacetate oxidized/β-hydroxybutyrate formed). That heart muscle, like other tissues (Snapper & Grünbaum, 1927, 1928; Mirsky & Broh-Kahn, 1937; Jowett & Quastel, 1935; Barnes, Drury, Greely & Wick, 1940; Wick & Drury, 1941), can oxidize ketone bodies has long been established qualitatively, especially by the work of Bing, Siegel, Ungar & Gilbert (1954) and Bing (1954–55). What has not been realized before is the preferential oxidation of the ketone bodies.

It may not be unexpected that sheep heart readily utilizes ketone bodies since in ruminants a large proportion of the foodstuff reaches the organs in the form of short-chain fatty acids or ketone bodies. But it is surprising that a non-ruminant heart also uses acetoacetate preferentially. Pigeon-heart homogenates behave similarly to rat heart (H. A. Krebs & L. V. Eggleston, unpublished work).

The observations on the effects of insulin differ from those reported by Beatty, Marco, Peterson, Bocek & West (1960) for striated muscle fibres of pancreas-diabetic rats. In this material insulin appears to accelerate acetoacetate removal. However, the experimental conditions and the type of tissue were very different and a comparison with the present results is not warranted.

Ketone bodies as fuels in vivo. In well-fed rats the concentration of ketone bodies in the blood is

below 0·5 mM. A concentration of 1 mM is regarded as mildly ketotic and in severe ketosis the value can rise to 20 mM (see Harrison & Long, 1940; Beatty *et al.* 1960). The data in Tables 2 and 3 therefore indicate that ketone bodies serve as fuel at concentrations that occur *in vivo*.

Formation of lactate. On addition of glucose plus insulin some lactate appeared in the medium. Acetoacetate also increased lactate formation and in the presence of acetoacetate insulin had no additional effect (Table 5). But this aerobic glycolysis was low (about 100 μmoles of lactate/g. dry wt./hr.) compared with the oxygen uptake (about 1600 μmoles of oxygen/g. dry wt./hr.). It was also low compared with the maximum rate of anaerobic glycolysis under the same conditions [2800 μmoles of lactate/g. dry wt./hr. (unpublished data; see also Morgan, Randle & Regen, 1959)]. The increased lactic acid production on addition of insulin supports the view that the rate of glucose entry into the cells may play some part in the control of the rate of glycolysis, i.e. the Pasteur effect (Randle & Smith, 1958; Morgan *et al.* 1959).

Ottaway & Sarkar (1958) also recorded an increased lactate formation in rat heart, perfused with acetoacetate, and in diaphragm, and Beatty *et al.* (1960) made similar observations on rat-muscle fibres. It is unlikely that lactate arises directly from acetoacetate. The effect is probably an indirect one. If acetoacetate suppresses the oxidation of glucose it leaves more intracellular glucose available for lactate formation. The stimulating effects of acetoacetate and of insulin on lactate formation have thus probably a common basis: both acetoacetate and insulin increase the amounts of intracellular glucose albeit by different mechanisms.

Effect of insulin. The fact that insulin in the absence of glucose decreased the oxidation of acetoacetate suggests that it has a direct stimulating effect on the combustion of endogenous material, besides its well established action on the entry of sugars. The values for the glycogen changes indicate that only part of the endogenous material made available for combustion by insulin is carbohydrate, and that insulin in the absence of glucose may increase the oxidation of substrates other than carbohydrates.

Inhibition of glucose uptake by acetoacetate. The slow uptake of glucose by the heart that occurred when insulin was not added was not affected by acetoacetate, but the rapid uptake of glucose in the presence of insulin was almost halved by 5 mM-acetoacetate. This observation is of relevance to the insulin assay based on the stimulation by insulin of the uptake of glucose by rat diaphragm (Groen, Kamminga, Willebrands & Blickman, 1952; Marsh & Haugaard, 1952; Randle, 1954, 1956; Vallance-Owen & Hurlock, 1954). Like rat heart, rat diaphragm oxidizes acetoacetate in preference to glucose (Krebs, Eggleston & d'Alessandro, 1961). If acetoacetate depresses the uptake of glucose in rat diaphragm as it does in rat heart, it would be expected to interfere with the insulin assay. Discrepancies noted by Steinke, Taylor & Renold (1961) in the assay of serum of untreated diabetics would then be accounted for by the presence of acetoacetate in this material.

SUMMARY

1. The removal of acetoacetate and glucose, the formation of β-hydroxybutyrate and lactate, the consumption of oxygen and the changes in the concentrations of glycogen were measured in the perfused heart preparation described by Bleehen & Fisher (1954). Acetoacetate, β-hydroxybutyrate, glucose and insulin, alone and in combinations, were added to the perfusate.

2. Acetoacetate was rapidly removed from the perfusate. At concentrations above 4 mM about half was recovered as β-hydroxybutyrate. At low concentrations (below 0·25 M) acetoacetate also disappeared rapidly but little was reduced to β-hydroxybutyrate. No intermediates of oxidation accumulated and the acetoacetate not accounted for as β-hydroxybutyrate may therefore be taken to be completely oxidized.

3. Added D(−)-β-hydroxybutyrate was as readily oxidized as acetoacetate.

4. Insulin with or without glucose somewhat lowered the rate of oxidation of acetoacetate. It is calculated from the rates of oxygen consumption and acetoacetate oxidation that in the absence of insulin acetoacetate (5 mM) contributed 73–82 % to the fuel of respiration. Insulin decreased this value to 51–64 %, in the presence or the absence of glucose.

5. Acetoacetate decreased the oxidation of glucose in the presence or in the absence of insulin. It halved the uptake of glucose by the heart in the presence of insulin.

6. Glucose when added as the sole substrate contributed 24 % to respiration and insulin raised this to 57 %. When acetoacetate and glucose were added, glucose contributed 4 % in the absence, and 15 % in the presence, of insulin.

7. The data indicate that acetoacetate is oxidized in preference to glucose and endogenous substrates.

8. Insulin increased the contribution made to respiration by endogenous substrates. This is taken to indicate that insulin has effects other than accelerating the entry of sugars into the tissue.

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Determination of Maltase and Isomaltase Activities with a Glucose-Oxidase Reagent

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Glucose oxidase is a valuable tool for the assay of glycosidase activities, especially when the substrate itself is a reducing sugar, and so the common reductometric methods cannot be used for estimation of the hydrolysis products. However, the commercially available glucose-oxidase preparations also attack maltose and isomaltose in addition to glucose, probably as an effect of contaminant glycosidases. These contaminating enzymes prohibit the use of glucose oxidase for the assay of maltase and isomaltase activities, and necessitate the use of more complicated methods (Dahlqvist, 1960*c*).

This paper describes a modification of the glucose-oxidase reagent so that it may also be used for the determination of maltase and isomaltase activities.

MATERIALS AND METHODS

Glucose-oxidase preparations. The following preparations were used: glucose oxidase 'crude' and glucose oxidase 'purified' (Sigma Chemical Co., St Louis, U.S.A.), glucose oxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), glucose oxidase–Dawes (obtained from Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A.), glucose oxidase pure (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), notatin Leo (Leo Pharmaceutical Prod., Copenhagen, Denmark) and glucose oxidase (C. F. Boehringer und Soehne, Mannheim, Germany).

Sugars. The commercial preparations used were of analytical-grade purity, glucose (dextrose) from Baker Chemical Co. (Phillipsburg, U.S.A.) and maltose monohydrate from Pfanstiehl Laboratories. Isomaltose was prepared in the laboratory by the method previously described (Dahlqvist, 1960*b*). α-Glucose refers to Baker Chemical Co. ordinary glucose (see above) dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}+112°$ (5% in water). β-Glucose was obtained from Nutritional Biochemicals Corp. The sugar was dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}+19°$ (5% in water). 'Equilibrium glucose' was obtained by keeping an appropriate solution of glucose at 20° for 18–20 hr. After that time $[\alpha]_D^{20}$ was +52·5° (10% in water). The solution

thus contained about one-third of the glucose present in the α-form and two-thirds in the β-form.

Other reagents. Ox-liver catalase was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) horseradish peroxidase from Worthington Biochemical Corp. and 2-amino-2-hydroxymethylpropane-1:3-diol (tris), buffer grade, from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Manometric determination of glucose-oxidase activity. The Warburg technique was used as described by Dixon (1951). Warburg flasks without centre cups were used. Each flask contained 2·0 ml. of acetate buffer–ethanol–catalase solution (50 mg. of ox-liver catalase and 1·0 ml. of 99·5% ethanol, made up with 0·1 M-sodium acetate–acetic acid buffer, pH 5·6, to a final volume of 100 ml.) and 1·0 ml. of an aqueous solution containing 9·85% (w/v) of equilibrium glucose. Into the side arm of each flask was introduced 0·5 ml. of glucose-oxidase solution containing 10–30 units of glucose oxidase. The reaction was performed at 25° in air; in oxygen the rate of oxygen uptake is 2·5 times that in air (Bentley, 1955). After equilibration the stoppers were closed and the flasks tipped. The absorption of oxygen was measured during 60 min. One unit of glucose-oxidase activity is the activity causing the consumption of 1 μl. of oxygen in 60 min. under these conditions.

The conditions used are essentially those recommended by Bentley (1955). The use of 0·1 M-β-glucose as substrate has been suggested, but the mutarotation of glucose then may cause difficulty since glucose oxidase does not oxidize α-glucose (Bentley, 1955). The author has therefore used 0·156 M-'equilibrium glucose' (which means 0·100 M-β-glucose and 0·056 M-α-glucose). Since α-glucose does not inhibit the oxidation of β-glucose (Bentley, 1955), this seemed to be a simple way to ensure the substrate concentration desired. The use of acetate buffer rather than phosphate buffer as suggested by Bentley (1955) does not alter the rate of oxidation (Keilin & Hartree, 1948).

Manometric determination of the maltase activity of glucose-oxidase preparations. Glucose formed by maltase in the presence of glucose oxidase will be oxidized to gluconic acid. For the determination of the maltase activity of glucose-oxidase preparations it was therefore impossible to use the reductometric method previously described (Dahlqvist, 1960*c*). For assay of the maltase activity of glucose-oxidase preparations a manometric method similar to that for determinations of glucose-oxidase activity was used, but the 'equilibrium glucose' solution was replaced by 1·0 ml. of 19·7% (w/v) maltose monohydrate in water, and the glucose-oxidase solution in the side arm of the flask contained 500–1000 units of glucose oxidase. The substrate solution was thus 0·146 M, pH 5·6, and the temperature 25°. The absorption of oxygen was measured during 60 min.

Under these conditions any glucose formed by the hydrolysis of maltose will be very rapidly oxidized, and each molecule of maltose hydrolysed will lead to the absorption of two molecules of oxygen. The relative maltase activity of the glucose-oxidase preparation is expressed as μl. of oxygen absorbed in 60 min. by 1000 units of glucose oxidase (see Table 1). Each 1 μl. of oxygen absorbed indicates the hydrolysis of 0·008 mg. of maltose. This way of expressing the maltase activity of glucose-oxidase preparations should not be confused with the unit for intestinal maltase activity specified below.

Spectrophotometric determination of glucose with α glucose-oxidase reagent. The method used was described by Huggett & Nixon (1957). The hydrogen peroxide formed during the dehydrogenation of glucose by glucose oxidase is catalysed by peroxidase at pH 7·0 to oxidize *o*-dianisidine to a brown compound. The intensity of the colour produced is measured spectrophotometrically.

The glucose-oxidase reagent of Huggett & Nixon (1957) was prepared in the following way: 125 000 units of glucose oxidase (i.e. 125 mg. of crude glucose oxidase, Sigma Chemical Co., or the corresponding amount of any other glucose-oxidase preparation; see Table 1), 0·5 mg. of horseradish peroxidase and 0·5 ml. of a 1% solution of *o*-dianisidine in 95% ethanol were taken and made up to 100 ml. with 0·5 M-sodium phosphate buffer, pH 7·0 [54·2 g. of $Na_2HPO_4,2H_2O$ and 23·4 g. of NaH_2PO_4 (or 26·9 g. of NaH_2PO_4,H_2O) dissolved in water and diluted to 1000 ml.; the pH was measured with a glass electrode and, if necessary, adjusted to 7·0]. The reagent was filtered through paper. On attempting to store this reagent in the refrigerator (at 4°) the phosphate buffer crystallized out.

For the determination of glucose, 0·5 ml. of the solution to be tested, containing 10–50 μg. of glucose, was mixed with 3·0 ml. of glucose-oxidase reagent in a test tube and placed in a water bath at 37° for 60 min. After that time the intensity of the colour produced was measured in a spectrophotometer at 420 mμ, with 1 cm. cuvettes, against a blank without sugar. At the same time a standard series was made, with tubes containing known amounts (10, 30 and 50 μg.) of glucose. The amount of glucose present should not exceed 50 μg., otherwise the Lambert–Beer law will not be obeyed.

The standard series may be prepared from a stock aqueous solution containing 100 mg. of glucose and 2·7 g. of benzoic acid in 1 l. This solution is stable for a long time at room temperature. The benzoic acid does not interfere with the enzymic determination of glucose.

Spectrophotometric determination of glucose with a modified tris–glucose-oxidase reagent. In this reagent the phosphate buffer has been replaced with 0·5 M-tris buffer, pH 7·0 (61·0 g. of tris is dissolved in 85 ml. of 5N-HCl and the solution is made up to 1 l. with water; the pH is measured with a glass electrode and, if necessary, adjusted to 7·0). Otherwise the preparation of the reagent and the determination of glucose are performed in the same way as described above for Huggett & Nixon's reagent.

The tris–glucose-oxidase reagent has an advantage over Huggett & Nixon's glucose-oxidase reagent, that it reacts considerably more slowly with maltose and isomaltose (see below). It may be stored for at least 3–4 days in the refrigerator.

RESULTS AND DISCUSSION

Glycosidase activities of different glucose-oxidase preparations

The analysis of a series of commercially available glucose-oxidase preparations (Table 1) showed them all to contain sufficient maltase activity to make them unsuitable for the determination of glucose in the presence of maltose. Isomaltose was hydrolysed, too, with a rate that was about one-third of that of maltose.

Many other disaccharides, however, such as turanose, palatinose (isomaltulose), lactose, cello-

Table 1. *Glucose-oxidase activity and relative maltase activity of some commercially available glucose-oxidase preparations*

Activities were measured manometrically, as described in the text.

Source and description	Glucose-oxidase activity (units/mg.)	Relative maltase activity (μl. of O_2 absorbed/1000 units of glucose oxidase)
Sigma Chemical Co; crude*	1 000	135
Sigma Chemical Co.; purified*	11 000	115
Worthington Biochemical Corp.*	1 000	135
Dawes (Pfanstiehl)*	10 000	50
Nutritional Biochemicals Corp.; pure*	17 600	113
Leo (Notatin Leo)†	18 600	180
C. F. Boehringer und Soehne†	13 850	35

* From *Aspergillus niger*.
† From *Penicillium notatum*.

biose, gentiobiose, sucrose and trehalose, were not hydrolysed by the glucose-oxidase preparations investigated, and therefore glucose oxidase has been used for the determination of the extent of hydrolysis of these disaccharides by glycosidases either by manometric (Dahlqvist, 1958, 1960*a*) or spectrophotometric (Dahlqvist, 1960*c*, 1961) methods.

That the action of glucose oxidase on maltose and isomaltose is caused by contaminating glycosidases rather than the oxidation of the disaccharides by glucose oxidase was shown by the fact that each molecule of maltose or isomaltose consumed two molecules of oxygen under the conditions used for manometric determinations, whereas one molecule of glucose consumed one molecule of oxygen. This fact also rules out the possibility that the oxygen consumption of maltose and isomaltose in the presence of glucose oxidase might be caused by contaminant glucose.

Attempts to separate glucose oxidase from the contaminant glycosidase activities

The purification of glucose oxidase from *Penicillium notatum* to yield a product free from maltase activity has been described by Coulthard *et al.* (1945). Attempts to use this procedure with one of the glucose-oxidase preparations studied (Worthington Biochemical Corp.) met with no success. Precipitation with tannic acid yielded a recovery of 30 % of the original glucose-oxidase activity, but lowered the relative maltase activity by only 25 %. Fractionation with ammonium sulphate (50–80 % saturation) yielded a recovery of 26 % of the glucose-oxidase activity, but did not lower the relative maltase activity at all. The negative results obtained may be explained by the fact that the enzyme preparations had been prepared from different species, since the Worthington Biochemical Corp. preparation is obtained from *Aspergillus niger*. The two glucose-oxidase preparations from *Penicillium notatum* (see Table 1) have not been subjected to fractionation by these methods.

Attempts to purify glucose oxidase by heat inactivation also gave negative results, and no encouraging results were obtained from preliminary experiments with cellulose ion-exchange columns (prepared and used as described by Peterson & Sober, 1956). Neither glucose oxidase nor maltase was adsorbed on carboxymethylcellulose in 0·01 M-maleate buffer, pH 5·3. Both enzymes were adsorbed on diethylaminoethylcellulose in 0·01 M-sodium phosphate buffer, pH 6·2, and they were eluted slowly and together by the addition of 0·10 M-NaCl to the buffer. The two enzymes thus seem to have similar chromatographic properties.

Selective inhibition of the glycosidase activities

Attempts were made to find an enzyme inhibitor which would inhibit the glycosidases without interfering with the glucose-oxidase activity. These experiments were performed with the spectrophotometric method of Huggett & Nixon (1957), with 50 μg. of glucose or 2 mg. of maltose (monohydrate) in each test tube. The substances to be tested for inhibitory action were added to the glucose-oxidase reagent. Tris buffer proved to be a suitable inhibitor.

The development of the colour with glucose and maltose as substrates in phosphate and tris buffers is seen in Fig. 1. When the reagent contained 0·5 M-phosphate buffer, pH 7·0 (i.e. the reagent of Huggett & Nixon 1957), intense colour was produced in 60 min. by 2 mg. of maltose, but when the buffer was replaced by 0·5 M-tris buffer, pH 7·0, the colour produced by maltose was strongly reduced. In the method for determination of the maltase activity of intestinal-glycosidase preparations which will be described below, each tube for glucose determination contains 1 mg. of maltose.

The development of colour with glucose was not significantly influenced by tris (Fig. 1). In other experiments, the presence of maltose did not influence the colour produced by varying amounts of glucose.

The isomaltase activity of glucose-oxidase preparations was found to be inhibited by tris in the same way as the maltase activity.

The selective inhibition of the glycosidase activities without interference with the glucose-oxidase activity has been demonstrated both with

Fig. 1. Development of colour with glucose and maltose by Huggett & Nixon's (1957) glucose-oxidase reagent, containing 0·5 M-phosphate buffer, pH 7·0, and a modified reagent in which the phosphate buffer has been replaced by 0·5 M-tris buffer, pH 7·0 (for preparation of the reagent see text). ●, Phosphate buffer; ○, tris buffer. The upper two curves were obtained with glucose (50 μg.) present; the lower two curves were obtained with maltose (2 mg.) present.

preparations obtained from *Aspergillus niger* and with those obtained from *Penicillium notatum*.

During the conditions for manometric determination of the glucose-oxidase and maltase activities of glucose-oxidase preparations the inhibitory effect of tris on the maltase activity seemed weaker. In the presence of 0·57 M-tris (12·11 g. of tris dissolved in about 50 ml. of water, adjusted with acetic acid to pH 5·6 and diluted with water to 100 ml., this buffer replacing the acetate buffer used elsewhere for the manometric determinations) the maltase activity was reduced by about 50 %, and the glucose-oxidase activity was not affected.

The weaker inhibitory action of tris on the maltase activity in the manometric determination might have been caused by the lower pH used (pH 5·6 in the manometric method and pH 7·0 in the spectrophotometric method). In an earlier study on the inhibition of intestinal glycosidases by tris only the non-ionized tris molecule was found to be an inhibitor (Dahlqvist, 1958), and thus the degree of inhibition increased with increasing pH. In the present case, however, when the manometric determination was performed at pH 7·0, the degree of inhibition by 0·57 M-tris was as low as at pH 5·6. Likewise, when the spectrophotometric method was used at pH 5·6, the degree of inhibition was as high as at pH 7·0. The inhibition of the maltase activity by tris thus seems to be independent of pH within this interval, and either the positively charged tris ion (which is the predominating species at both these pH values), or both the ion and the uncharged molecule, act as inhibitors.

The weaker effect obtained in the manometric method was caused by the higher maltose concentration in this method (0·146 M in the manometric, and 1·6 mM in the spectrophotometric method). Variation of the maltose concentration in the spectrophotometric method at pH 7·0 between 0·4 and 40 mM revealed that tris acted as a competitive inhibitor for the maltase activity, so that the degree of inhibition was dependent on the substrate concentration. K_s for the maltase activity (i.e. the substrate concentration yielding half-maximum velocity of hydrolysis) was determined to be approximately 1×10^{-2}, and K_i for tris as a competitive inhibitor (i.e. the dissociation constant for the enzyme–inhibitor complex) about 1×10^{-1} (the enzyme kinetic constants have been calculated as described by Dixon & Webb, 1958). Thus if the test tube under the conditions used for the enzymic determination of glucose contains less than 2 mg. of maltose (1·6 mM), the maltase activity will be inhibited by more than 80 % (cf. Fig. 1).

Influence of the mutarotation of glucose

Glucose oxidase catalyses the oxidation of only β-glucose (Keilin & Hartree, 1952*a*), whereas the glucose liberated from maltose and isomaltose by animal enzymes is probably α-glucose (Ben-Gershom & Leibowitz, 1958). The spontaneous mutarotation of glucose at neutral or slightly acid pH is a rather slow process (Pigman, 1957), but it is strongly catalysed by a specific enzyme, mutarotase, which is present in crude glucose-oxidase preparations (Keilin & Hartree, 1952*b*). This enzyme may be important for the determination of glucose with glucose oxidase, and the possibility that mutarotase may be inhibited by tris has to be considered.

Investigation of the spontaneous mutarotation of α-glucose under conditions similar to those used for colorimetric determination of glucose (i.e. in 0·5 M-tris buffer, pH 7·0) showed, however, that more than 80 % of the reaction was completed in 15 min. at 20°, and hence supposedly even more at 37°.

When the development of the colour of the tris–glucose-oxidase reagent with freshly prepared solutions of α-glucose and β-glucose (50 μg. of each) was studied it was found that both forms of glucose were oxidized at about the same rate. Thus although it cannot be decided from these experiments whether or not mutarotase is inhibited by tris, it is evident that both α- and β-glucose react rapidly with the tris–glucose-oxidase reagent.

Assay of intestinal maltase and isomaltase activities with the tris–glucose-oxidase reagent

In accordance with the general conditions previously specified for intestinal glycosidase-activity

determinations (Dahlqvist, 1960*c*), the enzyme should act for 60 min. at 37° on 0·028 M-substrate in the presence of 0·05 M-maleate buffer at optimum pH. One unit of glycosidase activity under these conditions is defined as that causing 5 % of hydrolysis in 2·0 ml. of reaction mixture.

The substrate solutions are prepared by dissolving 0·20 g. of maltose monohydrate or 0·19 g. of isomaltose in 0·1 M-maleate buffer, pH 6·5 (prepared as described by Gomori, 1955), to a final volume of 10·0 ml. A few drops of toluene are added as a preservative.

The enzyme solution to be analysed is diluted so as to contain 0·2–1·0 unit of maltase or isomaltase activity/ml. Of this diluted solution 0·1 ml. is transferred to a small test tube (7 mm. × 120 mm.). The tube is placed in a water bath at 37°, 0·1 ml. of substrate solution is added and the contents of the tube are mixed. After 60 min. of incubation, 0·8 ml. of water is added and the enzymic reaction is immediately stopped by immersing the tube in a boiling-water bath for 2 min.

A blank is prepared, which has the same composition, but in which the enzymic activity is stopped by boiling immediately after the mixing of enzyme and substrate.

After the tubes are cooled with tap water, their contents are well mixed and 0·5 ml. is transferred to another test tube (16 mm. × 150 mm.). To each tube is added 3·0 ml. of the tris–glucose-oxidase reagent (prepared as described above) and the tubes are placed in a water bath at 37° for 60 min. for development of the colour. A standard series with glucose (see above) is prepared for each set of determinations. The intensity of the colour is measured in a spectrophotometer at 420 mμ in 1 cm. cuvettes, against a sample without sugar.

The production of 50 μg. of glucose during these conditions corresponds to 1 unit of maltase or isomaltase activity/ml. of the diluted enzyme solution used for the determination. The degree of hydrolysis then is 5 %.

Use of the tris–glucose-oxidase reagent for assay of other intestinal-glycosidase activities

The tris–glucose-oxidase reagent may replace the Huggett & Nixon (1957) reagent in the method described for the assay of intestinal-turanase, -lactase, -cellobiase and -gentiobiase activity (Dahlqvist, 1960*c*), as well as for measuring the activity towards non-reducing substrates such as sucrose and trehalose. The number of glucose molecules liberated per substrate molecule must, however, be taken into account, since glucose oxidase does not react with fructose or galactose (Dahlqvist, 1960*c*).

This reagent thus may be used as a general tool for measuring glycosidase activities which liberate glucose from a disaccharide or an oligosaccharide. It is not suitable, however, for the assay of phenylglucosidase activities, since free phenol interferes with the production of the colour (Dahlqvist, 1960*c*).

SUMMARY

1. Commercially available glucose-oxidase preparations contain also maltase and isomaltase activity. Attempts to separate glucose oxidase from these contaminating glycosidase activities were unsuccessful.

2. The presence of 0·5 M-tris buffer, pH 7·0, selectively inhibits these glycosidase activities, without interfering with the production of colour with free glucose by a glucose-oxidase reagent containing *o*-dianisidine as a chromogen.

3. A method for the determination of intestinal maltase and isomaltase activities based upon a tris–glucose-oxidase reagent is described.

4. The tris–glucose-oxidase reagent may be used as a general reagent for the determination of glucose liberated by glycosidases from di- and oligo-saccharides. It is not suitable for the assay of the hydrolysis of phenylglucosides.

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Sterol Metabolism

6. THE INTERCONVERSION OF CHOLESTEROL, 7-DEHYDROCHOLESTEROL AND LATHOSTEROL IN THE RAT*

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The three closely related sterols, cholesterol, 7-dehydrocholesterol and lathosterol, occur in animal tissues in different proportions. From their structures they would appear to be interconvertible by simple dehydrogenation or reduction steps as shown in Fig. 1, but unequivocal evidence for such a mechanism has not yet been obtained.

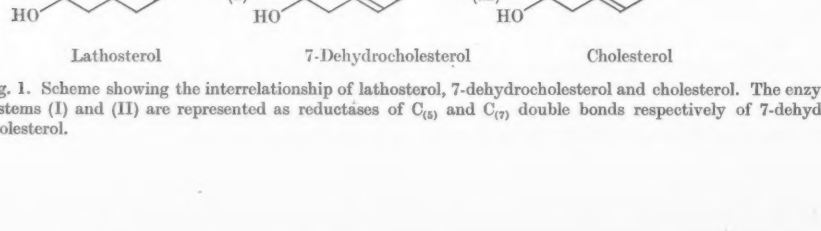
It has generally been assumed that 7-dehydrocholesterol (provitamin D) is formed *in vivo* by dehydrogenation of cholesterol, and observations on the feeding of cholesterol to guinea pigs supported this concept (Glover, Glover & Morton, 1952). The work was done, however, before the discovery of lathosterol, which is difficult to separate from its isomer cholesterol. It has been shown that lathosterol can be converted into cholesterol *in vivo* (Cook, Kliman & Fieser, 1954) and *in vitro* (Frantz, Davidson, Dulit & Mobberley, 1959; Mercer & Glover, 1959), but the intermediates have not yet been established. The formation of cholesterol from zymosterol (Johnston & Bloch, 1957) and lathosterol (Frantz *et al.* 1959) requires aerobic conditions, which has tentatively been interpreted as perhaps involving hydroxylation followed by a dehydration step. This process would not easily be reversible. The rapid and extensive reduction of 7-dehydrocholesterol to lathosterol that takes place in the guinea pig and in the rat (Glover & Green, 1957; Glover & Stainer, 1959) implies that direct reduction of the $C_{(5)}$ double bond occurs very readily in both species. It is perhaps simplest therefore for the present to assume that a reductase works reversibly. Evidence for the reduction of the $C_{(7)}$ bond of provitamin D to give cholesterol was not clear-cut in the same balance studies. The equivocal result for the latter reduction possibly arises because (*a*) it proceeds relatively slowly compared with the absorption of the product, and (*b*) the normal exchange and mixing of endogenous cholesterol and dietary sterols during absorption always result in sweeping some endogenous sterols into the lymph. Consequently a more critical experiment on the metabolism of [4-^{14}C]cholesterol in the rat has been carried out.

Other experiments with pure preparations of lathosterol, 7-dehydrocholesterol and cholesterol were also undertaken to determine if these three sterols were easily interconvertible in the rat. First, changes in the mucosal sterols of the small intestine were followed during the absorption of cholesterol and lathosterol in turn. Secondly, the interconversion of the various sterols was studied *in vitro* by using everted sacs of rat intestine.

* Part 5: Glover, Green & Stainer (1959).

EXPERIMENTAL

Materials. Cholesterol (British Drug Houses Ltd.) was purified by the dibromide technique of Schwenk & Werthessen (1952). The product was crystallized twice from



Lathosterol 7-Dehydrocholesterol Cholesterol

Fig. 1. Scheme showing the interrelationship of lathosterol, 7-dehydrocholesterol and cholesterol. The enzyme systems (I) and (II) are represented as reductases of $C_{(5)}$ and $C_{(7)}$ double bonds respectively of 7-dehydrocholesterol.

methanol. 7-Dehydrocholesterol (cholesta-5:7-dienol) (Peboc Ltd., Liverpool) was freshly crystallized from methanol as required. Lathosterol (cholest-7-enol) was prepared from 7-dehydrocholesterol by the method of Schenck, Buchholz & Wiese (1936). Last traces of 7-dehydrocholesterol were removed from the product by chromatography on MgO with 2% (v/v) diethyl ether in light petroleum (b.p. 40–60°) as the eluent. The lathosterol was crystallized several times from methanol. The crystals melted at 123° and had the correct i.r. spectrum, and the u.v. spectrum showed no residual 7-dehydrocholesterol. [4-^{14}C]Cholesterol (22 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, and stored at 0° in benzene solution. Before use it was diluted with pure cholesterol, and the resulting mixture purified from any radiation decomposition products by the dibromide procedure as above.

Animals. Male rats (wt. 150–250 g.) were selected and separated into uniform groups of four litter mates or animals of same weight and age. They were maintained on the cube diet no. 4 (British Extracting Co. Ltd., Port Sunlight). Each group of rats was placed on a diet of lipid-free food cubes (the above diet exhaustively extracted with ether) for 4 or 5 days before the start of the experiment. The rats were starved for 24 hr. before being dosed with sterol. In the lathosterol experiment 27 mg. was given to each rat but in the cholesterol experiments doses of 50 mg. were used. The cholesterol was suspended in the minimum of arachis oil (0·5 ml./rat) and mixed with a small amount of extracted food cubes (3 g./rat). The rats always ate the whole of the dose within 30 min. of its being presented. They and the controls were killed 3 hr. later with $CHCl_3$ and the small intestines were excised for sterol analysis. The animals from which intestinal sacs were prepared were killed by a blow on the back of the neck.

In the experiments with [4-^{14}C]cholesterol, four male rats were dosed with 10 mg. each (0·43 μc/mg.).

Preparation of everted intestinal sacs. These were prepared by the method of Wilson & Wiseman (1954). The length of small intestine (measured from the pyloric sphincter) taken from each animal in the control and test groups was the same. In Expts. 9–14 the sacs were incubated in the medium used by Smith, Hauk & Treadwell (1958), the sterols being held in suspension by means of bovine serum albumin (5 mg./ml.). The sacs were filled and slightly distended with the medium minus the albumin, sterol and glucose. The sacs were incubated for 3 hr. at 37°. A gas mixture of $O_2 + CO_2$ (95:5, v/v) was passed over the incubation mixture, which was shaken throughout at a rate of 60 excursions/min.

Isolation of intestinal mucosal lipids. The small intestine was washed with 0·9% NaCl, cut open and placed on a glass surface cooled with salt–ice mixture underneath, and the mucosa scraped off with a blunt scalpel. The everted intestinal sacs from experiments *in vitro* were treated similarly at the end of the incubation period. In a few experiments the lipids were extracted directly from the mucosa by the procedure described by Glover, Goodwin & Morton (1947); however, in the majority of cases the mucosa was saponified and the non-saponifiable matter extracted as follows. Mucosa (x g.) was refluxed for 1 hr. with x ml. of 0·25% pyrogallol in ethanol and $\frac{1}{2}x$ ml. of aq. 60% KOH. The mixture was then cooled, diluted with 3 vol. of distilled water and extracted five times with dry peroxide-free ether. The bulked extracts were washed free of alkali with water, dried over anhydrous Na_2SO_4 and evaporated under N_2. This saponification process has no effect on the sterols and does not alter the relative proportions of 'slow-acting' to 'fast-acting' sterols.

Extraction of sterols from non-saponifiable matter in experiments with ^{14}C. The 3β-hydroxy sterols were separated from the non-saponifiable material by precipitation as their digitonides (Windaus, 1909) and regenerated by the method of Schoenheimer & Dam (1933).

Chromatographic separation of 'slow-acting' cholesterol from 'fast-acting' sterols in experiments with [4-^{14}C]*cholesterol.* The mixed sterols were esterified with azobenzene-4-benzoyl chloride [4-(phenylazo)benzoyl chloride] and chromatographed on a column of silicic acid–Celite 503 (2:1, w/w), with light petroleum–benzene (10:1, v/v) for development of the chromatogram (Idler & Baumann, 1952). When the two orange zones had completely separated the column was run dry and extruded. The zones were then cut out and extracted with benzene–ethanol (50:50, v/v). The upper zone consists of the azo esters of the 'fast-reacting' sterols (mainly lathosterol and 7-dehydrocholesterol) and the lower zone is the cholesteryl azo ester. The free sterols were regenerated from their azo esters by the saponification method of Idler & Baumann (1952).

Estimation of sterols. The 'slow-reacting' and 'fast-reacting' sterols were assayed by the method of Moore & Baumann (1952). The extinctions were read in the Unicam SP. 600 visual spectrophotometer at 620 mμ. 7-Dehydrocholesterol was determined spectrophotometrically by taking the u.v.-absorption spectra of either the lipid, or unsaponifiable matter or mixed sterol in ethanol and correcting for irrelevant absorption with the Morton & Stubbs (1946) three-point correction procedure. The wavelengths selected were 276·5, 281·5 and 298 mμ, and the corresponding extinctions E_1, E_2 and E_3 respectively were recorded fo reach extract. Then $E_{corr.}$ at 281·5 mμ = 0·654 × $(5E_2 - 3E_1 - 2E_3)$. The u.v. readings were made on a Unicam SP. 500 spectrophotometer. The amount of lathosterol in a sterol mixture was obtained by deducting the value for 7-dehydrocholesterol obtained from the spectrum from the value for total 'fast' sterol by the Moore & Baumann method.

Radioassay. Non-saponifiable materials, azo esters and sterols were prepared by pipetting 0·2 ml. of a $CHCl_3$ solution of the material on to a stainless-steel planchet (1·5 cm. diam.), the base of which was covered with a disk of lens paper (Green's no. 105). The solvent was evaporated by irradiation from above with i.r. lamps (250 w). The lens paper ensures a uniform distribution of material over the planchet. The material was assayed to $< 5\%$. The usual corrections for background, self-absorption and coincidence were made. The geometrical efficiency of the end-window counter was 5% with a background of 10–12 counts/min.; that of the proportional gas-flow counter was 50%.

RESULTS

Experiments in vivo. The relative amounts of the three sterols found in the intestinal mucosa of control rats and of animals dosed with cholesterol (Expts. 1–3) and lathosterol (Expts. 4–6) are shown in Table 1. The final columns in the table give the

Table 1. *Interconversion of sterols in rat intestinal mucosa*

The dose was 50 mg. of cholesterol or 27–29 mg. of lathosterol/rat. Results are expressed in mg. of sterol from the mucosa of two rats in each group. Δ^7, Lathosterol; $\Delta^{5:7}$, 7-dehydrocholesterol; Δ^5, cholesterol.

Expt. no.	Sterol fed	Controls (*a*)			Dosed (*b*)			Difference (*b* − *a*)		
		Δ^7	$\Delta^{5:7}$	Δ^5	Δ^7	$\Delta^{5:7}$	Δ^5	Δ^7	$\Delta^{5:7}$	Δ^5
1	Cholesterol	0·20	0·08	6·6	0·17	0·01	14·5	−0·03	−0·07	+7·9
2	—	0·40	0·02	9·0	0·28	0·02	15·2	−0·12	0	+6·2
3	—	0·54	0·04	13·3	0·23	0·01	17·7	−0·31	−0·03	+4·4
4	Lathosterol	0·74	0·05	15·4	6·77	1·40	15·2	+6·03	+1·35	−0·2
5	—	0·37	0·04	11·3	5·24	0·43	10·0	+4·87	+0·39	−1·3
6	—	0·49	0·05	22·3	6·97	1·15	23·1	+6·48	+1·10	+0·8

Table 2. *Conversion of* [4-^{14}C]*cholesterol into* [4-^{14}C]*cholest-7-enols in the rat* (*Expt. no.* 7)

The number of rats used was 4.

Sterol fraction	Amount (mg.)	Specific activity (counts/min./mg.)
Total administered	40·2	47 104
Total recovered from mucosa	42·4	9 640
Cholesterol	41·5	9 740
Total cholest-7-enols	0·8	5 953

differences between the test and control groups. Although the cholesterol levels of the mucosa from rats absorbing this sterol have risen by 30–120% there was an appreciable drop in the lathosterol and 7-dehydrocholesterol levels instead of an increase by enzymic conversion of the additional cholesterol.

The absorption of lathosterol, however, raised the level of this sterol in the mucosa 10- to 15-fold and at the same time brought about a 10- to 25-fold increase in the 7-dehydrocholesterol content above normal. About 10–20% of the absorbed lathosterol was oxidized to 7-dehydrocholesterol. There was no significant or consistent change in the cholesterol levels, so no definite conclusion can be drawn about its possible formation in these experiments.

In order to follow the behaviour of cholesterol more carefully, a new experiment was carried out with ^{14}C-labelled material. [4-^{14}C]Cholesterol was administered to one group of four rats and the various sterols in the musosa were examined as before. The results are presented in Table 2.

The 'fast-reacting' sterol fraction acquired radioactivity, indicating that some of the material fed was converted into the cholest-7-enols. The specific activity of this fraction (0·8 mg.), however, is only two-thirds of that of the much larger cholesterol pool (42 mg.) in the mucosa. The rate of conversion or turnover of cholesterol into the 'fast-reacting' sterols must therefore be very slow. The amount of 7-dehydrocholesterol in the rat intestine was unfortunately too small in these experiments to permit separation for accurate radioassay.

Experiments in vitro. To avoid the complication of the loss of sterols from the small intestine by the normal absorption process, a number of experiments were also carried out *in vitro* with everted sacs. The results for the incubation of 7-dehydrocholesterol, lathosterol and cholesterol, dispersed in the medium with bovine serum albumin, are shown in Tables 3 and 4. During the 3 hr. incubations, 50–80% of the various sterols added were absorbed by the mucosa despite the absence of bile salts. This is perhaps seen more readily in Table 4, where the composition of the sterols in the residual medium of Expts. 9 and 10 is also given.

The additional lathosterol in Expts. 11–13 produced only a small increase in the amount of 7-dehydrocholesterol but a marked rise in cholesterol. Whether the cholesterol is synthesized in this experiment via 7-dehydrocholesterol or by another route is uncertain, but the results are consistent with the 7-dehydrocholesterol route. Indeed, Expts. 8–10 show quite clearly that 7-dehydrocholesterol is readily reduced to both isomers, cholesterol and lathosterol, in approximately equal amounts. Cholesterol supplements (Expt. 14), however, cause no detectable changes in the other sterols.

DISCUSSION

The above results confirm previous observations in this Laboratory that some of the 7-dehydrocholesterol undergoing absorption is readily reduced in the mucosa to the more saturated sterols cholesterol and lathosterol. They extend them in that both steps (I and II in Fig. 1) have now been shown to be reversible. It is proposed to refer to the enzymes concerned as the 5-ene and 7-ene reductases respectively of cholesta-5:7-dienol since the steady-state equilibrium in the unfed rat favours the more saturated sterols. The term 'cholesterol dehydrogenase', used in previous papers for the enzyme acting on the 7:8 double

Table 3. *Interconversion of sterols by everted intestinal sacs of the rat*

Each sterol was added in a 25 ml. aqueous dispersion (approx. 1 mg./ml.) with bovine albumin. Results are expressed as mg. of sterol from the mucosa of two rats' intestines in each group: Δ^7, Lathosterol; $\Delta^{5:7}$, 7-dehydrocholesterol; Δ^5, cholesterol.

Expt. no.	Sterol added	Controls (*a*)			Dosed (*b*)			Difference (*b* − *a*)		
		Δ^7	$\Delta^{5:7}$	Δ^5	Δ^7	$\Delta^{5:7}$	Δ^5	Δ^7	$\Delta^{5:7}$	Δ^5
8	7-Dehydrocholesterol	0·12	0·04	3·17	4·68	5·31	6·35	+4·56	+5·27	+3·18
9	—	0·22	0·05	2·55	6·82	4·36	9·03	+6·60	+4·31	+6·48
10	—	0·15	0·08	4·34	6·05	6·50	8·78	+5·90	+6·42	+4·44
11	Lathosterol	0·08	0·07	6·00	15·70	0·08	10·67	+15·6	+0·01	+4·67
12	—	0·09	0·06	6·15	15·70	0·07	11·00	+15·6	+0·01	+4·85
13	—	0·26	0·12	5·28	19·19	0·17	7·75	+18·9	+0·05	+2·47
14	[4-^{14}C]Cholesterol (9·8 mg., 12·9 μc)	0·27	0·09	12·30	0·26	0·09	20·15 (9·2 μc)	−0·003	+0·003	+7·85

Table 4. *Uptake of sterols from aqueous medium by everted intestinal sacs*

The initial medium was Krebs & Henseleit (1932) bicarbonate buffer (pH 7·4) containing 1 mg. of sterol/ml. dispersed with bovine albumin. Incubation period, 3 hr.

Expt. no.	Sterol added to medium	Quantity (mg.)	Group	Cholesterol (mg.)	7-Dehydrocholesterol (mg.)	Lathosterol (mg.)	Total sterols (mg.)	Uptake (%)
9	7-Dehydrocholesterol	0	Control	2·55	0·05	0·22	2·82	—
		26·4	Test	9·03	4·36	6·82	20·21	66
			Final test-medium	4·71	1·36	0·59	6·66	—
10	7-Dehydrocholesterol	0	Control	4·34	0·08	0·15	4·57	—
		23·9	Test	8·78	6·50	6·05	21·33	70
			Final test-medium	1·40	0·20	0·30	1·90	—

bond (Glover *et al.* 1952), is now regarded as unsuitable since it has also been used for the enzyme catalysing the oxidation of the 3β-hydroxy group.

5-Ene reductase. With regard to the 5-ene reductase, experiments both *in vivo* and *in vitro* show clearly that the reduction of a single dose of 7-dehydrocholesterol to lathosterol can take place rapidly. ('Single dose' is emphasized here because unpublished observations indicate that as the absorption of 7-dehydrocholesterol is prolonged over 24 hr. the capacity of the mucosal system to reduce it to lathosterol becomes much smaller.) The results for the oxidation of lathosterol, however, are slightly different *in vitro* compared with *in vivo*. Whereas the ratio of lathosterol to 7-dehydrocholesterol in the mucosa of the unfed rat lies in the range 10–12 (see Table 1), in animals dosed with lathosterol this was lowered slightly to 5–12 in spite of a more than tenfold increase in the absolute amount of lathosterol present in the tissue. Thus, *in vivo*, the oxidation of lathosterol to 7-dehydrocholesterol takes place readily. However, *in vitro*, 3–6 hr. after incubation of the control everted sacs, the above ratio declined to 1–4, mainly owing to a lower lathosterol content (Table 3). In the presence of added lathosterol, the ratio rises to 100–200 with only a slight increase in the 7-dehydrocholesterol. The cholesterol level on the other hand rises markedly. This suggests that any 7-dehydrocholesterol formed is immediately reduced in the 7-position to cholesterol. The low concentration of 7-dehydrocholesterol may reflect a less efficient state of aeration of the mucosa in the isolated sac compared with that in the normal intestine. The requirement for oxygen in the transformation of lathosterol into cholesterol has been observed by Frantz *et al.* (1959).

7-Ene reductase. The above results from experiments on the administration of cholesterol to rats and work with 7-dehydrocholesterol (Glover & Stainer, 1959) suggest that, if a 7-ene reductase is active, the amount of 7-dehydrocholesterol formed from cholesterol and vice versa was either too small to be detected or else it merely replaced endogenous material swept into the lymph by the normal exchange process that occurs during absorption. The fact that the formation of cholesterol from 7-dehydrocholesterol is readily detected *in vitro* implies that the latter explanation is probably correct. At least, the reduction of 7-dehydrocholesterol to cholesterol can take place, although apparently less readily than to lathosterol *in vivo*.

When [4-^{14}C]cholesterol was administered to rats, it was possible to demonstrate the formation of the corresponding 4-^{14}C-labelled 'fast-reacting'

sterols but their combined specific activity is two-thirds of that of the cholesterol, which is present in 52 times their total amounts (Table 2). The oxidation of about 0·5 mg. of cholesterol back to the 'fast-reacting' sterols would account for the above result. Thus the reaction takes place only slowly. A possible explanation is that only a portion of the cholesterol is exposed to the enzyme. Previous work, however, shows that the exchange or turnover of the various sterols with one another in the cell is so rapid that this should not really be considered a serious limiting factor in cholesterol oxidation. Similarly, *in vitro*, cholesterol is not readily converted into the 'fast-reacting' sterols.

The evidence as a whole suggests that conditions in the cell favour the formation of cholesterol from lathosterol via 7-dehydrocholesterol rather than the reverse reactions, so the 'fast-reacting' sterols are precursors of cholesterol rather than metabolites. This may not, however, be the only biosynthetic route for the formation of cholesterol, for desmosterol (cholesta-5:25-dienol) can also be converted into cholesterol (Stokes, Hickey & Fish, 1958).

The above sequence, however, is in keeping with the work of Brooks & Baumann (1957) with [2-^{14}C]-acetate and our own recent work with [2-^{14}C]-mevalonate, a preliminary account of which has been published (Mercer & Glover, 1959).

Intestinal mucosa homogenates prepared by the Bucher procedure (Bucher & McGarrahan, 1956) were tried several times for their capacity to interconvert the various sterols, but their activity was invariably low although they can synthesize sterols from [^{14}C]acetate or [^{14}C]mevalonate. Frantz *et al.* (1959) also appeared to have difficulty with liver homogenates in studying the same reaction.

All the sterols suspended in the bovine serum albumin were well absorbed by the intestinal sacs without the addition of bile salts. Thus the main function of the latter in sterol absorption is probably due to their dispersing action.

That true absorption across the cell membrane is occurring and not simple adsorption on it is indicated by the fact that in Expts. 8–10 the absorbed 7-dehydrocholesterol is reduced to lathosterol. This reaction does not occur under the above conditions when the cell membranes are broken. In fact, the results for the sterol composition of the residual medium, after incubation, indicate that some cholesterol and lathosterol secretion has taken place concomitantly with 7-dehydrocholesterol absorption.

SUMMARY

1. 7-Dehydrocholesterol and lathosterol are readily interconverted in the intestine of the rat *in vivo* and *in vitro*. The equilibrium of the enzyme system favours lathosterol. The relative ease with which this reaction takes place indicates that the enzyme involved may be a reductase, which has been tentatively called '7-dehydrocholesterol 5-ene reductase'.

2. A little 7-dehydrocholesterol is simultaneously reduced to cholesterol in the same tissue but at a much lower rate than the above interchanges. The back reaction, cholesterol to 7-dehydrocholesterol, proceeds even more slowly. The enzyme system responsible for this step has been tentatively called '7-dehydrocholesterol 7-ene reductase'.

3. The above results imply that the cholest-7-enols are precursors of cholesterol rather than metabolites.

4. Each of the sterols dispersed in bovine-albumin solution was readily absorbed by everted intestinal sacs *in vitro* without the addition of bile salts. This shows that the sterol-absorption system is not completely dependent on bile.

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Chromatographic Separation of Brain Lipids

2. ETHANOLAMINE-CONTAINING PHOSPHOLIPIDS*

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There have been several previous reports on the chromatographic behaviour of the ethanolamine-containing phospholipids on columns of alumina (Lea, Rhodes & Stoll, 1955; Rhodes & Lea, 1957) and silicic acid (Hanahan, Dittmer & Warashina, 1957; Rhodes & Lea, 1957; Macfarlane, Gray & Wheeldon, 1960). However, the lipid compositions of the two sources of ethanolamine-based phospholipid which have been most extensively studied, namely liver and egg yolk, present a relatively less complicated problem than brain tissue does.

It has been our experience with brain-lipid extracts that ethanolamine-containing phospholipids are not readily separated from phosphatidylserine, cerebroside and sulphatide on silicic acid columns; on alumina columns the elution patterns of ethanolamine-based phospholipids and sulphatide overlap. Egg yolk appears to be free from cerebroside and nearly free from phosphatidylserine; there have been no reports on its sulphatide content, if any. Liver seems to be the only animal tissue that is virtually free from cerebroside (Williams *et al.* 1945), although it does contain phosphatidylserine and there is some evidence for the presence of sulphatide (Green & Robinson, 1960).

In an earlier paper (Long & Staples, 1961) in which a brain-lipid extract was used, we reported the separation of cerebroside from aminophospholipid by gradient-elution chromatography on an alumina column. Continued gradient elution has now been found to produce a peak containing the whole of the ethanolamine-based phospholipid, completely free from phosphatidylserine, which latter remained firmly adsorbed on the alumina under these conditions. The eluted ethanolamine-containing lipid was, however, contaminated with sulphatide. Rechromatography of the ethanolamine-containing fractions on silicic acid gave a peak containing only a mixture of phosphatidylethanolamine and ethanolamine plasmalogen. A further fraction containing lysophosphatidylethanolamine and sulphatide could later be eluted.

A preliminary report of some of this work has already been published (Long & Staples, 1960).

* Part 1: Long & Staples (1961).

EXPERIMENTAL

Purification of solvents, specifications of silicic acid and of alumina (type *B*), preparation of rat-brain-lipid extracts, general chromatographic procedures and analytical methods for galactose, sulphate, amino nitrogen, total nitrogen and total phosphorus were as described by Long & Staples (1959, 1961). Acyl ester was determined by the hydroxamate procedure of Shapiro (1953), by using purified ovolecithin (Rhodes & Lea, 1957) as a standard, with the assumption of an acyl ester:phosphorus ratio of 2·0:1; the fatty acid:phosphorus ratio of the ovolecithin had the theoretical value of 2·0:1.

Additional analytical methods

Long-chain aldehydes. A sample of lipid, containing not more than the equivalent of 0·08 μmole of aldehyde, was decomposed with 90% (v/v) acetic acid and treated with Schiff's reagent. The procedure used was a modification of the method of Leupold & Büttner (1953), devised by Dr G. H. Sloane-Stanley of this Department (personal communication).

Potassium. The lipid sample containing not more than 0·15 μg.atom of potassium, was evaporated to dryness in a Pyrex-glass test tube and then ashed at 170° for 2 hr. with 0·1 ml. of 72% $HClO_4$. After dilution with water to 3 ml., the potassium content was determined with a flame photometer (Evans Electroselenium Ltd.) with a standard (0·15 μg.atom of K^+) and a blank subjected to the same procedure.

Ethanolamine and serine. The principle of Axelrod, Reichenthal & Brodie (1953) was used with several modifications.

The lipid sample, containing not more than 0·4 μmole of ethanolamine or 0·5 μmole of serine, was evaporated to dryness in a glass-stoppered 10 ml. centrifuge tube, and 0·5 ml. of 6N-HCl was added. The stoppered tube was heated on a boiling-water bath for 2 hr., cooled and treated with 0·8 ml. of methanol and 1·6 ml. of $CHCl_3$. The contents were mixed thoroughly with a Pasteur pipette and the lower $CHCl_3$-rich phase, containing fatty acids, fatty aldehydes, sphingosine and cholesterol, was withdrawn and discarded. The aqueous methanolic phase was washed twice in the same way with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The final aqueous methanolic phase was then evaporated to dryness in a current of air or N_2 on a warm-water bath.

The dry residue was dissolved in water (1·2 ml.) and treated with 0·3 ml. of aqueous 5% (w/v) $NaHCO_3$ and 0·1 ml. of a 5% (v/v) solution of 1-fluoro-2:4-dinitrobenzene in ethanol. The tube was heated on a water bath

at 75–80° for 75 min. After cooling, 4 ml. of $CHCl_3$ was added to the mixture. The tube was then vigorously shaken and centrifuged. A 1 ml. sample of the upper aqueous phase was taken for the determination of serine, and a 3 ml. sample of lower phase for ethanolamine.

The $CHCl_3$ phase (3 ml.), containing dinitrophenylethanolamine, was evaporated to dryness in a current of air. The residue was dissolved in 4 ml. of 6N-HCl and extracted once with 5 ml. of light petroleum (b.p. 40–60°), the upper phase being discarded. The extinction of the strongly acidic lower phase was measured at 420 mμ in a Unicam SP. 1400 prism absorptiometer, and compared with a standard (0·4 μmole of ethanolamine) and a blank subjected to the same procedure.

The aqueous phase (1 ml.), containing dinitrophenylserine, was acidified with 0·5 ml. of N-HCl, and shaken with 5 ml. of *iso*butyl methyl ketone. After centrifuging, 4 ml. of the upper phase was transferred to another 10 ml. glass-stoppered centrifuge tube, to which was then added 4 ml. of 0·1N-NaOH. The tube was shaken vigorously and centrifuged; 3 ml. of the lower alkaline phase was removed and treated with 0·5 ml. of 2N-HCl. The extinction of this solution was also read at 420 mμ, and compared with a standard (0·5 μmole of serine) and a blank subjected to the same procedure.

RESULTS

Chromatography of rat-brain lipids on alumina

The washed protein-free lipid from three rat brains (about 5 g. fresh wt.), dissolved in 25 ml. of chloroform–methanol (50:50, v/v), was applied to a column of alumina (13 g.; 17 cm. × 1 cm. diam.; type *B*, Long & Staples, 1961), equilibrated with the same solvent mixture. The column was run with chloroform–methanol (50:50, v/v) and three 25 ml. fractions were collected, in which were present the cholesterol and choline-containing phospholipids. Gradient elution was then begun. The mixing vessel contained 125 ml. of chloroform–methanol (50:50, v/v), and 150 ml. of chloroform–methanol–water (45:45:10, by vol.) was placed in the reservoir. Eleven 25 ml. fractions were collected. The water content of the eluting solvent rose from 0 to 7 % (by vol.), and the fractions contained all the cerebroside and most of the sulphatide present in the lipid sample. The first traces of amino nitrogen appeared in fraction 12 (Long & Staples, 1961). A second gradient was then applied. The mixing vessel contained 125 ml. of chloroform–methanol–water (46·5:46·5:7, by vol.), and 200 ml. of chloroform–methanol–water (43:43:14, by vol.) was placed in the reservoir. Nine more 25 ml. fractions were collected, the water content of the eluting solvent increasing from 7 to 12·5 % (by vol.).

Each fraction was analysed for amino nitrogen, total nitrogen, ethanolamine, serine, total phosphorus, acyl ester, aldehyde, galactose and sulphate. Some of the analytical values for fractions 9–23 in a typical experiment are shown in Fig. 1. Amino-nitrogen analyses (not shown), by the ninhydrin method (Moore & Stein, 1948), were always made first, and gave an indication of the amounts of aminophospholipid present in the fractions. These values served as a guide for calculating the volume of each fraction to be taken for the exact determination of the other constituents. In Fig. 1 the discontinuous curve on the left shows the trailing edge of the elution of cerebroside, details of which are to be found in Fig. 4 of Long & Staples (1961).

In the individual fractions comprising the aminophospholipid elution curve (fractions 12–23), close agreement was found for the contents of ethanolamine, total phosphorus and total nitrogen, except in the very early fractions where the total-nitrogen values were high because of the simultaneous elution of sulphatide. When allowance was made for the nitrogen content of the sulphatide (calculated from the sulphate determinations), summation of the individual analytical values for fractions 12–23 gave the following data: total phosphorus, 106·1 μg.atoms; total nitrogen, 103·9 μg.-atoms; ethanolamine, 101·3 μmoles. The crude lipid applied to the column contained 116 μmoles of ethanolamine (i.e. 26·3 μmoles/g. of fresh brain), indicating a recovery of 88 %. In three similar experiments, the ethanolamine recoveries were 89, 99 and 100 %. In no case has any serine been found

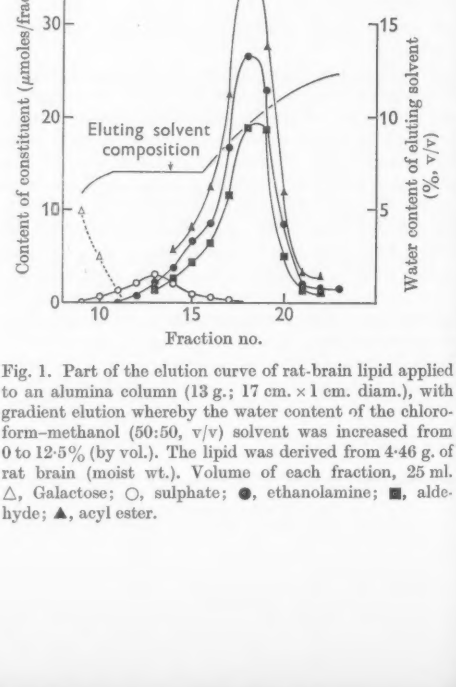

Fig. 1. Part of the elution curve of rat-brain lipid applied to an alumina column (13 g.; 17 cm. × 1 cm. diam.), with gradient elution whereby the water content of the chloroform–methanol (50:50, v/v) solvent was increased from 0 to 12·5% (by vol.). The lipid was derived from 4·46 g. of rat brain (moist wt.). Volume of each fraction, 25 ml. △, Galactose; ○, sulphate; ●, ethanolamine; ■, aldehyde; ▲, acyl ester.

in these fractions, although in the experiment recorded in Fig. 1 the serine content of the lipid applied to the column was 35·2 μmoles (i.e. 7·9 μmoles/g. of fresh brain). This observation accords with the equivalence of total nitrogen and ethanolamine contents.

The acyl ester:phosphorus ratio for fractions 12–23 averaged 1·22:1, and the mean aldehyde:phosphorus ratio was 0·68:1. However, there were variations between experiments, aldehyde:phosphorus ratios from 0·48:1 to 0·70:1 having been obtained. For a mixture of phosphatidylethanolamine and ethanolamine plasmalogen, the theoretical value for the ratio (aldehyde + acyl ester):phosphorus is 2·0:1. In the experiment shown in Fig. 1, the observed average value for this ratio was 1·90:1, indicating the presence of lyso compounds. In the early fractions, however, it sometimes exceeded 2·0:1, suggesting the presence of some additional acyl ester. It may therefore be concluded that, although the bulk of the material eluted in the aminophospholipid peak consists of a mixture of phosphatidylethanolamine and ethanolamine plasmalogen, the fractions also contain some sulphatide and ethanolamine lysophospholipid as well as some 'excess ester'.

Chromatography on silicic acid of the rat-brain ethanolamine-containing lipids eluted from an alumina column

Silicic acid chromatography of the ethanolamine-containing fractions from the alumina column, by means of gradient elution with increasing content of methanol in chloroform, seemed likely to separate the 'excess ester' and lysophosphatidylethanolamine as fast- and slow-running fractions respectively from the mixture of phosphatidylethanolamine and ethanolamine plasmalogen, but it appeared probable that the separation of sulphatide from the latter two substances might present a more difficult problem. We have already reported (Long & Staples, 1961) that, on silicic acid, sulphatide is somewhat less readily eluted than are the ethanolamine-based phospholipids. However, it was first necessary to determine whether the sulphatide should be applied to the silicic acid column in the form of the free acid or as the potassium salt in order to effect the better separation. To test this point, those fractions eluted from an alumina column that contained ethanolamine-based lipid together with the bulk of the sulphatide, the latter in the free acid form (Long & Staples, 1961), were combined and evaporated to dryness. The residue was dissolved in chloroform–methanol (98:2, v/v) and divided into two equal portions.

One sample, which was untreated and in which the sulphatide was present as the free acid, was chromatographed directly on silicic acid (8 g.; 17 cm. × 1 cm. diam.) by gradient elution, whereby the content of methanol in the chloroform was raised from 2 to 14% (by vol.). The result of this experiment is shown in Fig. 2 (*a*).

The other portion was treated with methanol (0·5 vol.) and 0·1 M-potassium chloride (0·3 vol.). The mixture was shaken and centrifuged, and the upper aqueous methanolic phase was discarded. The lower phase was washed twice with an equal volume of methanol–0·1 M-potassium chloride–chloroform (48:47:3, by vol.). In this way the sulphatide was converted into its potassium salt. Finally the chloroform-rich lower phase was evaporated to dryness, and the residue was dissolved in chloroform–methanol (98:2, v/v) and chromatographed on silicic acid, with the same conditions as described for the untreated portion. The result of this experiment is shown in Fig. 2 (*b*).

Figs. 2 (*a*) and 2 (*b*) show that the elution pattern of the ethanolamine-based lipids is practically the same in both cases, as would be expected. However, the elution of sulphatide was more difficult to

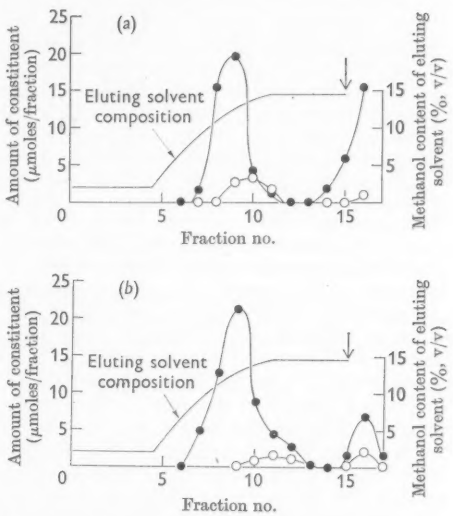

Fig. 2. Silicic acid chromatography of lipid material obtained from fractions 12–23 eluted from an alumina column of the type shown in Fig. 1. Gradient elution was used whereby the methanol content of the chloroform–methanol solvent was increased from 2 to 14% (by vol.). At arrow (fraction 15), the solvent was changed to methanol–chloroform–water (70:25:5, by vol.). Dimensions of silicic acid column: 8 g.; 17 cm. × 1 cm. diam. Volume of each fraction, 25 ml. ●, Ethanolamine; ○, sulphate. The sulphatide component was applied as (*a*) free acid, (*b*) potassium salt.

achieve when the latter was applied to the column as the potassium salt (Fig. 2*b*). In both cases the main sulphatide peak eluted during the application of the gradient was free from potassium. When the solvent was changed after fraction 15 to methanol–chloroform–water (70:25:5, by vol.), a small additional amount of sulphatide was eluted in each case, together with the ethanolamine-containing lysophospholipid. In the experiment of Fig. 2 (*b*), potassium was also eluted in this fraction, and was equivalent in amount to the total quantity of sulphatide applied to the column.

In view of these results, it appeared probable that sulphatide, applied as the potassium salt, would not be eluted from a silicic acid column by a solvent containing up to 9% (v/v) of methanol in chloroform, whereas the phosphatidylethanolamine and ethanolamine plasmalogen would be eluted completely under these conditions. Accordingly, a mixture of ethanolamine-containing lipid and sulphatide, obtained from an alumina column, was washed with aqueous potassium chloride as described for the experiment of Fig. 2 (*b*). The lipid was dissolved in chloroform–methanol (98:2, v/v) and applied to a silicic acid column. After elution of the 'excess ester' in two 25 ml. fractions with chloroform–methanol (98:2, v/v), gradient elution was applied in which the mixing vessel contained 125 ml. of chloroform–methanol (98:2, v/v), and 150 ml. of chloroform–methanol (88:12, v/v) were placed in the reservoir. In this way the methanol content of the eluting solvent was raised from 2 to 9% (by vol.), and after this solvent had passed through the column a further quantity of chloroform–methanol (91:9, v/v) was run in, until 14 more 25 ml. fractions had been collected. An ethanolamine-containing peak was eluted (Fig. 3). This peak was free from sulphatide, and contained in fractions 9–14 the following amounts of constituents: ethanolamine, 37·9 μmoles; total phosphorus, 40·4 μg.atoms; total nitrogen, 36·4 μg.-atoms; ester, 53·1 μmoles; aldehyde, 28·5 μmoles; indicating a mixture of phosphatidylethanolamine and ethanolamine plasmalogen in the ratio of about 30:70. Further, analyses of the individual fractions showed that the composition of the eluted material was constant. In fractions 15 and 16, some ethanolamine-containing lysophospholipid was eluted, as judged from the (acyl ester + aldehyde): phosphorus ratio of 1·02:1.

When the solvent composition was changed after fraction 16 to chloroform–methanol (80:20, v/v), 7·06 μmoles of sulphate were eluted in the next seven fractions, out of 7·87 μmoles applied to the column. This material was accompanied by the remainder of the ethanolamine-containing lysophospholipid (ethanolamine, 6·64 μmoles; total phosphorus, 6·83 μg.atoms; ester, 6·00 μmoles; aldehyde, 1·51 μmoles). Out of 48·4 μmoles of ethanolamine applied to the silicic acid column, 46·4 μmoles were recovered, i.e. 96%.

Stability of ethanolamine-containing phospholipids on an alumina column

In early experiments with alumina columns, the usual practice was to elute the cerebroside on the first day and the ethanolamine-containing lipids on the following day. Under these conditions, the first fraction collected on the second day consistently showed an abnormally high content of acyl ester (acyl ester:phosphorus ratio, approx. 6:1), although the contents of total phosphorus, total nitrogen, ethanolamine and aldehyde were as expected. This suggested that some type of lipid decomposition had occurred during storage for 17 hr. on the alumina column. In order to test the stability of the ethanolamine-containing lipids under these conditions, a sample of the mixture of phosphatidylethanolamine and ethanolamine plasmalogen, prepared as described in the last section, and containing 65 μmoles of ester and 31·8 μmoles of aldehyde, was dissolved in chloroform–methanol (50:50, v/v) and applied to an alumina column (13 g.). At intervals during 6 weeks, the column was eluted with chloroform–methanol (50:50, v/v)

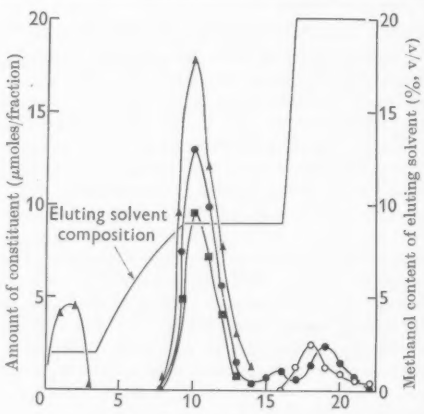

Fig. 3. Silicic acid chromatography of lipid material obtained from fractions 12–23 eluted from an alumina column of the type shown in Fig. 1. The sulphatide component was applied as the potassium salt. Gradient elution was used whereby the methanol content of the chloroform–methanol solvent was increased from 2 to 9% (by vol.). After fraction 16, the solvent was changed to chloroform–methanol (80:20, v/v). Dimensions of silicic acid column: 8 g.; 17 cm. × 1 cm. diam. Volume of each fraction, 25 ml. ▲, Acyl ester; ●, ethanolamine; ■, aldehyde; ○, sulphate.

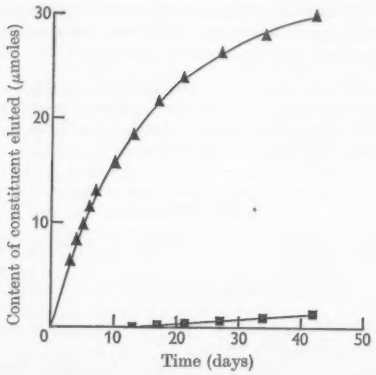

Fig. 4. Decomposition of a mixture of purified phosphatidylethanolamine and ethanolamine plasmalogen on a column of alumina (13 g.; 17 cm. × 1 cm. diam.). The total content of constituent (ordinate) is calculated by summation of the individual quantities eluted on the days of analysis (abscissa). Lipid applied to column contained 65 μmoles of acyl ester and 31·8 μmoles of aldehyde. ▲, Acyl ester; ■, aldehyde.

and a 25 ml. fraction was collected; the 'hold-up volume' of the column was 8 ml. When these fractions were analysed, no phosphorus was found, but acyl ester was present in measurable quantity in all cases. After about 2 weeks a trace of aldehyde was also eluted. The results are shown in Fig. 4.

Subsequent elution of the column with increasing content of water from zero up to 14 % (by vol.) in the chloroform–methanol (50:50, v/v) solvent led to the very slow elution of ethanolamine-containing lipid which was not further characterized.

It will be seen that, although the ethanolamine-containing lipids do decompose on an alumina column, the rate is quite low.

DISCUSSION

In the work described, no attempt has been made to separate phosphatidylethanolamine from ethanolamine plasmalogen. The possibility of effecting this separation chromatographically seems rather remote, since the difference in properties due to the replacement of one acyl group by an $\alpha\beta$-unsaturated ether group would probably be no greater than that due to differences in chain length or degree of unsaturation of those component fatty acids which are present in the phosphatidylethanolamine. The ethanolamine-containing lipids isolated from rat brain in several individual experiments have shown aldehyde:phosphorus ratios varying from 0·48:1 to 0·70:1; the ester:phosphorus ratios varied in an inverse manner.

The isolation of the ethanolamine-containing phospholipids from the lipid mixture present in brain falls into two parts, namely separation from (*a*) cerebroside and phosphatidylserine, and (*b*) sulphatide. On an alumina column, gradient elution with increasing content of water in chloroform–methanol (50:50, v/v) has been found effective in achieving the separation from cerebroside (see also Long & Staples, 1961). The presence of phosphatidylserine unexpectedly presented no difficulty, since this substance is very firmly bound to alumina (Long, Shapiro & Staples, 1960) and is not easily extracted from it. The ethanolamine-containing phospholipid eluted from an alumina column is contaminated with sulphatide and also with small amounts of 'excess ester' produced by lipid decomposition (see below) as well as lyso compounds.

Separation of the mixture of phosphatidylethanolamine and ethanolamine plasmalogen from the aforementioned contaminants was achieved on a silicic acid column, since the 'excess ester' was readily eluted with chloroform–methanol (98:2, v/v), while the sulphatide and lysophosphatidylethanolamine were rather strongly retained.

The source of the 'excess ester' was at first attributed to the breakdown of some of the ethanolamine-containing lipid on the alumina column. However, a later experiment with purified material (Fig. 4) showed that the ethanolamine-containing lipids were fairly stable under these conditions unless there was very prolonged contact. We believe that the nature of the decomposition is most simply expressed by the following transesterification:

Phosphatidylethanolamine + methanol →
lysophosphatidylethanolamine + methyl ester.

This conclusion is largely based on the behaviour of phosphatidylserine (Long *et al.* 1960, and unpublished observations), which is extremely labile in contact with alumina.

It may be calculated from Fig. 4 that in 2-day experiments the quantity of 'excess ester' produced by breakdown of the ethanolamine-based phospholipids would not exceed 3·3 % of the amount present in these lipids, whereas in a 1-day experiment it would amount to about 1 %. The average values observed for 'excess ester' were 38·8 and 16·7 % respectively. These results are consistent with the likelihood that the 'excess ester' is in fact largely derived from the decomposition of phosphatidylserine.

Since the decomposition scheme for the ethanolamine-containing phospholipids referred to in the last paragraph probably takes place to a very limited extent only under the chromatographic conditions employed in 1-day experiments on an alumina column, this mechanism cannot be held

responsible for the appreciable quantity of lysophosphatidylethanolamine found to be present. Although it is possible that some breakdown of phosphatidylethanolamine occurs during the process of evaporating the fractions from the alumina column in preparation for silicic acid chromatography, this seems unlikely to be extensive. It is probable therefore that lysophosphatidylethanolamine is a normal constituent of rat brain, and is not an artifact produced by experimental manipulations.

SUMMARY

1. Chromatography of mixed rat-brain lipids on an alumina column by a gradient method, in which the water content of a chloroform–methanol solvent was raised from 7 to 12·5% (by vol.), resulted in the elution of a peak containing all the ethanolamine-based phospholipids.

2. The main components were phosphatidylethanolamine and ethanolamine plasmalogen, but smaller quantities of sulphatide, lysophosphatidylethanolamine and 'excess ester' were also present.

3. Chromatography of this partially purified material on silicic acid, whereby the methanol content of a chloroform–methanol solvent system was gradually raised from 2 to 9% (by vol.), produced a satisfactory separation into the following fractions: (*a*) 'excess ester'; (*b*) a mixture of phosphatidylethanolamine and ethanolamine plasmalogen; (*c*) a mixture of sulphatide and lysophosphatidylethanolamine.

4. Phosphatidylethanolamine and ethanolamine plasmalogen have been found to be moderately stable when in contact with alumina.

We wish to express our thanks to Dr G. H. Sloane-Stanley for making available his unpublished procedure for aldehyde determination, and to Dr G. C. R. Morris for help with the potassium determinations.

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The Effect of Staphylococcal Leucocidin on the Leucocyte

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Staphylococcal leucocidin consists of two proteins that act synergistically to kill the leucocytes of humans and rabbits (Woodin, 1960). Observations on the cytotoxicity of leucocidin have been restricted to the use of simple viability tests such as the ability of leucocidin to inhibit respiration or produce morphological changes in susceptible cells. The present paper describes some biochemical changes in the leucocyte treated with leucocidin.

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EXPERIMENTAL

Materials

Leucocytes. These were obtained from peritoneal exudates 5–10 hr. after injection of 0·85% NaCl. The exudate, to which heparin (10 mg./1000 ml.) was added, was filtered through surgical gauze and centrifuged at 200*g* for 5 min. The cells were then washed three times by centrifuging at 200*g* for 5 min. in Hanks medium or a Krebs-Ringer solution (see below) and suspended in one of these media, and a sample was taken for counting. The data in this paper, unless otherwise stated, refer to rabbit leucocytes.

Macrophages. These were taken from the peritoneal cavity 4–5 days after the injection of 80 ml. of liquid paraffin. They were then treated in the same way as the leucocytes. The data in this paper, unless otherwise stated, refer to rabbit macrophages.

Suspension media for the leucocytes and macrophages. According to the conditions of the experiment the media used were: Hanks medium (Hanks, 1948); Krebs–Ringer bicarbonate (Umbreit, Burris & Stauffer, 1957); Krebs–Ringer bicarbonate from which phosphate was omitted; Hanks medium containing 0·5% of gelatin or Krebs–Ringer bicarbonate containing 10% of dextran (Dextran 10; Pharmacia, Uppsala, Sweden). Unless otherwise recorded, to all these media glucose (2 mg./ml.) was added.

Leucocidin. The two components of leucocidin were crystallized by the methods of Woodin (1960) and stored at 4° in saturated $(NH_4)_2SO_4$. Before use the suspended solid was dissolved in, and dialysed against, 0·85% NaCl, pH 5–6. Solutions of either component, about 2 mg./ml., stored at 4° have shown no change in activity over several weeks.

Streptolysin O. This was provided by Professor A. Bernheimer and stated to contain about 50 000 haemolytic units/mg. Before use it was dissolved in 0·1 M-cysteine–HCl in Hanks medium, adjusted to pH 7·2.

Adsorption of leucocidin by the leucocyte

Leucocytes were suspended in 0·5% gelatin in Hanks medium and tubes set up containing various amounts of cells. A constant amount [9·6 μg. of *S* ('slow') component, 8·4 μg. of *F* ('fast') component] of one or both of the leucocidin components was added, and the mixtures were made up to 9 ml. with 0·5% of gelatin in Hanks medium and incubated for 10 min. at 37°. The suspensions were centrifuged at 650*g* for 5 min. and the supernatants assayed for the two components of leucocidin by the Neisser & Wechsberg method (Woodin, 1959). When the *F* or the *S* component of leucocidin was added separately the assay could be carried out directly on the supernatant. When both components were present it was necessary to dialyse the supernatant for 6 hr. against 0·85% NaCl to remove reducing material.

Investigation of the leucocidin effects

Incubation conditions. Suspensions of leucocytes, 10^6–10^7/ml., were incubated at 37° for 10 min., usually one sample containing leucocidin and one not. Unless otherwise recorded each component was added to give 2 μg./10^8 cells. Test tubes were usually used and the cells kept evenly suspended by gentle rocking.

Homogenization of cells. After incubation the suspensions were centrifuged for 5 min. at 650*g* in graduated tubes and the supernatant was removed from the cell pellet. The pellets were suspended in about 5 ml. of homogenization medium and disrupted by treatment for 3 min. in the Mullard ultrasonic generator (Measuring and Scientific Equipment Ltd., Crawley, Sussex). The tubes were kept in ice during this treatment. For homogenization in Potter–Elvehjem tubes the cell pellets were mixed with an equal volume of the homogenization medium and homogenized for 10 min., the tubes being cooled in ice. Microscopic examination showed 100% cell breakage under these conditions. The homogenate was then diluted to 10 times the original volume of the cell pellet and centrifuged at 110 000*g* for 45 min. at 0°. In this way three fractions were obtained: the cell supernatant, the soluble fraction of the homogenate and the insoluble fraction of the homogenate.

Nucleic acids of the leucocyte. After incubation of the cells in Hanks medium, the three fractions were prepared as described, homogenization being carried out in Hanks medium with Potter–Elvehjem tubes. The method of Schneider (1945) was used to isolate the nucleic acids. The ribonucleic acid in the extract was estimated by the orcinol reaction (Ceriotti, 1955), and the deoxyribonucleic acid by the diphenylamine reaction (Burton, 1956). The standard in the orcinol reaction was a sample of yeast nucleic acid (British Drug Houses Ltd., Poole, Dorset) which was partially purified by the method of Kunitz (1941) and contained 8·9% of P. The standard in the diphenylamine reaction was a sample of thymus deoxyribonucleic acid, prepared by the method of Kay, Simmons & Dounce (1952), which contained 9·2% of P.

Phosphoprotein of the leucocyte After incubation of the cells in Hanks medium the cell suspensions were cooled in ice and ice-cold trichloroacetic acid was added to give 10% (w/v). The phosphoprotein (determined as alkali-labile P) was estimated by the method of Schmidt & Thannhauser (1945) except that extraction with chloroform–methanol (2:1, v/v) was substituted for extraction with ethanol and ether.

Lipids of the leucocyte. After incubation of the cells in Hanks medium the cell suspensions were centrifuged at 2600*g* for 15 min. and the supernatant was removed. Chloroform–methanol (20 vol.; 2:1, v/v) was added and the suspension left 4–5 hr. at room temperature. The suspension was filtered and the filtrate washed with 2 mM-$CaCl_2$ by the method of Folch, Lees & Sloane-Stanley (1957). The chloroform-soluble fraction was dried in a rotary evaporator and the solids were dissolved in chloroform. For analysis of the lipid constituents samples were dried in a rotary evaporator. For the determination of dry weight the sample was subsequently dried *in vacuo* over P_2O_5. For separation of the phospholipids samples of the chloroform solution containing 50–90 μg.atoms of P were passed down a column containing 10 g. of silicic acid in chloroform (Mallinkrodt A.R.) maintained at 4°. The column was washed with about 100 ml. of chloroform and eluted with chloroform–methanol (9:1, v/v) and then with chloroform–methanol (2:3, v/v). Fractions were collected and the phosphorus content was determined.

Respiratory mechanisms of the leucocyte and macrophage. (*a*) *Glycolysis in whole cells.* Duplicate suspensions of leucocytes or macrophages (10^7/ml.) were incubated in glucose-free Hanks medium for 15 min. at 37°, one suspension containing leucocidin. A sample (0·5 ml.) was taken from each suspension and deproteinized with an equal volume of trichloroacetic acid (10%, w/v). Glucose (2 mg./ml.) and KCN (50 μg./ml.) were then added to the remaining cell suspensions and incubation was continued at 37° for 30 min., when a further sample (0·5 ml.) of each suspension was deproteinized. The lactic acid present in the deproteinized solutions was then determined (Barker & Summerson, 1941).

(*b*) *Glycolysis in homogenates.* Homogenates of normal and leucocidin-treated leucocytes were prepared in Hanks medium in Potter–Elvehjem tubes and diluted with 0·154 M-KCl. Mixtures were then set up containing, in 3 ml.,

150 μg. of KCN, an amount of homogenate corresponding to $0{\cdot}4 \times 10^8$ cells and the cofactors and substrates given by Umbreit *et al.* (1957) as suitable for the measurement of glycolysis in liver homogenates. A third mixture (3 ml.) containing homogenate from leucocidin-treated leucocytes contained also 1 mg. of *F* component of leucocidin and 1 mg. of *S* component of leucocidin. Samples of the mixtures (1 ml.) were deproteinized immediately after adding the homogenate and after incubation for 30 min. at 37°. The lactic acid in the deproteinized solution was determined. Preliminary experiments indicated that lactate production was proportional to the amount of homogenate added when that was less than the equivalent of 10^8 cells.

(*c*) *Oxygen uptake in whole cells.* Leucocytes or macrophages were suspended in Krebs–Ringer phosphate solution and O_2 uptake was measured in Warburg manometers, with 2 ml. suspensions, 10^8 cells/ml. After about 20 min. leucocidin was tipped in from the side arm and measurement continued for a further 60 min.

Water, Na^+ and K^+ ion contents of the leucocyte and macrophage. Cells suspended in Hanks medium (2 ml. containing 10^8 cells) were incubated in weighed tubes and then centrifuged at 650*g* for 4 min. The supernatant was removed, and the tubes were drained, weighed, dried at 110° and weighed again. The dried residue was ashed in HNO_3 and the Na^+ and K^+ ion contents were determined by flame photometry (Amoore, Parsons & Werkheiser, 1958).

Acid-soluble constituents of the leucocyte. After incubation in phosphate-free, glucose-free Krebs–Ringer bicarbonate solution the cell suspensions (12 ml. containing 4×10^9 cells) were cooled in ice and made 0·5M with respect to $HClO_4$. Extraction was continued for 30 min. at 0° and the suspensions were then centrifuged at 80 000*g* for 1 hr. The supernatant was removed and analysed. The $HClO_4$ was removed when necessary, as the potassium salt. For some experiments the cell suspensions, after incubation, were centrifuged at 300*g* for 5 min., and the supernatants and cell residues separately treated with $HClO_4$. In calculating the amount of an acid-soluble constituent the anhydrous volume of material precipitated by $HClO_4$ was neglected. In the estimation of the ninhydrin-reacting material glycine was used as a standard.

For paper chromatography of the ninhydrin-reacting material in the acid extracts of the cell residues and supernatants, these were separated after incubation and extracted with trichloroacetic acid (final concn. 10%, w/v) in the same way as for the $HClO_4$ extraction. The trichloroacetic acid was then extracted into ether and the water-soluble fraction passed down a column of Zeo-Karb 225 in the H^+ form (Permutit Co. Ltd., London) with about 5 ml. of resin for 10 ml. of extract. The columns were washed free from Cl^- ions and then eluted with 0·5N-NH_3 solution. The eluate before and for 25 ml. after the appearance of free NH_3 in the eluate was then freeze-dried. The solid was dissolved in a small volume of water and applied to Whatman no. 1 paper, and a chromatogram developed with butanol–acetic acid–water (60:10:40, by vol.). The paper was sprayed with ninhydrin in butanol.

Non-diffusible material liberated from leucocytes by leucocidin. After incubation of the cells in Hanks medium (30 ml. containing 10^{10} cells) a sample (10 ml.) of the supernatant from leucocidin-treated cells was isolated and dialysed for several days against distilled water, and the suspension of precipitated material dried at 110° in a weighed ampoule. The N content of the dry solid was then determined. Further samples (2 ml.) of the leucocidin-treated leucocyte supernatant were analysed for P, cholesterol, fatty acid and esterified fatty acid. For electrophoresis and ultracentrifuging a sample (5 ml.) of the supernatant of leucocidin-treated cells was dialysed against 0·1M-NaCl–0·02M-tris buffer, pH 7·5.

Distribution of protein in the leucocyte and macrophage. After incubation of the cells in Hanks medium (30 ml. containing 10^{10} cells) the cell supernatant and the soluble fraction and insoluble fraction of the homogenate were prepared. The three fractions were precipitated and washed with 10% (w/v) trichloroacetic acid. The extraction with trichloroacetic acid was followed by extraction with ethanol (10 ml.), and it was apparent that much protein dissolved in the ethanol. After extraction with ethanol the nucleic acids were dissolved by heating for 15 min. in 5% (w/v) trichloroacetic acid (10 ml.) at 90°. The N contents of the material soluble in ethanol and the material insoluble in ethanol and insoluble in hot trichloroacetic acid were then determined. The protein contents were calculated from the sum of these values, assuming these proteins to contain 16% of N. The N contents of the fractions will contain a contribution from the N-containing lipids. This is very small (see below) and was the same for both normal and leucocidin-treated leucocytes.

To prepare solutions for analytical ultracentrifuging, the cell pellets of 10^{10} normal and 10^{10} leucocidin-treated leucocytes were each homogenized in 0·3M-sucrose in Potter–Elvehjem tubes. The homogenates were diluted to 10 ml. with 0·3M-sucrose and the insoluble fraction of the homogenate was centrifuged down. The supernatants were then dialysed against 0·1M-NaCl–0·02M-tris buffer, pH 7·5.

Lysozyme and phagocytin of the leucocyte. After incubation the leucotye suspensions (5 ml. containing $2 + 10^9$ cells) were centrifuged at 650*g* for 5 min. and the supernatants separated. The cell pellets were then homogenized and the sucrose-soluble and citric acid-soluble fractions prepared by the method of Hirsch (1960). The lysozyme and phagocytin contents were then determined by the methods used by Hirsch (1960). Lysozyme contents are given as unit weights of egg-white lysozyme. Phagocytin units are the dilutions at the end points.

Readsorption of the proteins present in the leucocidin-treated leucocyte supernatant. After incubation of leucocytes in Hanks medium the cell supernatants were isolated. The insoluble fractions of normal and leucocidin-treated leucocytes homogenized ultrasonically in Hanks medium were then prepared and washed three times in Hanks medium by centrifuging at 36 000*g* for 30 min. at 0°. They were then diluted in Hanks medium to the original volume of the cell suspensions. Tubes were then set up with constant volumes (containing 3·5 mg. of protein) of the leucocidin-treated cell supernatant and various volumes of the washed insoluble residues of the homogenized cells. After mixing, the tubes were left at room temperature for 10 min. and centrifuged at 36 000*g* for 30 min. at 0°. The trichloroacetic acid-insoluble N of the supernatants was then determined.

Analytical methods

Chemical methods. These were: reducing sugar, Park & Johnson (1949); amino N, Yemm & Cocking (1955); lipid-bound amino N, Lea & Rhodes (1954); esterified fatty

acid, Morgan & Kingsbury (1959); fatty acid, Dole (1956); cholesterol, Henly (1957); free and total aldehyde, Gray & Macfarlane (1958); hexose diphosphate, hexose monophosphate, adenosine diphosphate and adenosine triphosphate, Slater (1953); inorganic P, Berenblum & Chain (1938). For the analysis of the acid extracts of leucocytes the inorganic P was precipitated as the calcium salt (Delory, 1938). The inorganic P was also determined, without precipitation as the calcium salt, but after incubating the extract of the leucocyte for 30 min. at room temperature in the acid–molybdate reagent of the Berenblum & Chain (1938) method. The difference between the values for the inorganic P determined by calcium precipitation and that found after incubation in the acid–molybdate reagent was taken as the creatinine phosphate content. Acid-labile P was determined as the inorganic P liberated after hydrolysis at 100° in N-H_2SO_4 for 10 min. For determination of organically-bound P samples were digested in conc. H_2SO_4–60% (w/v) $HClO_4$ (3:2, v/v). N was determined by micro-Kjeldahl.

Analytical ultracentrifuging. This was done in a Spinco model E ultracentrifuge.

Electrophoresis. This was done in an Antweiler electrophoresis apparatus.

RESULTS

Adsorption of the two components of leucocidin by the leucocyte. Fig. 1 shows that each component of leucocidin is adsorbed by rabbit leucocytes. When both components are present there is greater adsorption of both, indicating that there is no competition for the sites at which adsorption occurs. When saturated with each component leucocytes contain 1 μg./10^8 cells, corresponding to 10^5 molecules/cell. No adsorption on guinea-pig leucocytes could be detected. The insensitivity of guinea-pig leucocytes to the lethal effects of leucocidin has been noted by Gladstone & van Heyningen (1957).

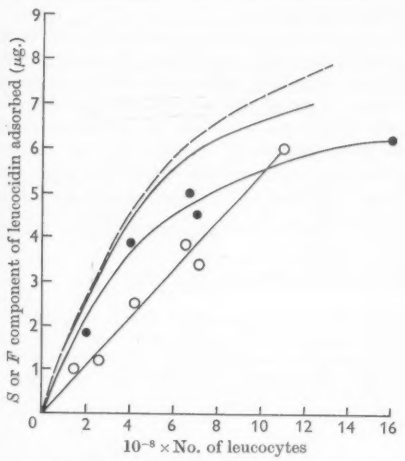


Fig. 1. Adsorption of leucocidin by rabbit leucocytes. ○, *F* component; ●, *S* component; – – –, *S* component in the presence of the *F* component; —, *F* component in the presence of the *S* component. The experimentally determined points for the adsorption of each component in the presence of the other have been omitted. The amounts of the *F* and *S* components added were 8·4 μg. and 9·6 μg. respectively in a total volume of 9 ml.

Tests for enzymic activity in the leucocidin components. Solutions containing the *F* or the *S* component of leucocidin (1 mg./ml.) and a solution containing both components at this concentration were found to be free from the following activities: ribonuclease (Kunitz, 1941), deoxyribonuclease (Laskowski & Seidel, 1945), staphylokinase (Bungay, 1961), staphylococcal haemolysin (Glenny & Stevens, 1935), egg-yolk turbidity-producing factor (Gillespie & Adler, 1952). On incubation with casein (2·0% in 0·85% NaCl, pH 7·5), no inorganic P was liberated. On incubation with ribonuclease (0·5% in Hanks medium) there was no increase in amino N. Neither component hydrolysed diphosphopyridine nucleotide, reduced diphosphopyridine nucleotide or triphosphopyridine nucleotide (Dr R. van Heyningen, unpublished results). It is known that many of these activities are present in some staphylococcal filtrates (Elek, 1959), and their absence from leucocidin is evidence for the homogeneity of the two components.

Effect of leucocidin on some structural components of the leucocyte. Only the insoluble fraction of homogenized leucocytes contained nucleic acid, and the values (ribonucleic acid, 0·15 mg./10^8 cells and deoxyribonucleic acid, 0·6 mg./10^8 cells) were identical for both normal and leucocidin-treated cells. Thus no degradation of nucleic acid accompanies leucocidin action. Similarly, the phosphoprotein-P content (0·08 μmole/10^8 cells) was the same in normal and leucocidin-treated cells. Table 1

Table 1. *Effect of leucocidin on the content of some acid-soluble constituents of the leucocyte*

After incubation in glucose-free, phosphate-free Krebs–Ringer solution the leucocyte suspensions were treated with $HClO_4$ before analysis.

Material	Amount in extract of 10^8 leucocytes: Normal	Leucocidin-treated
Reducing sugar (μg. of glucose)	44	46
Nitrogen (μg.)	34	39
Ultraviolet-absorbing material in 0·5 N-$HClO_4$ (vol. × $E^{1\,cm.}_{260}$)	4·0	4·2
Orcinol-reacting material (μg. of ribonucleic acid)	95	106
Phosphorus (μg.)	34	33
Ninhydrin-reacting material (μg. of glycine)	110	110

gives the concentration of some of the acid-soluble constituents of suspensions of normal and leucocidin-treated leucocytes and, as no significant differences exist between the two sets of values, provides more evidence for absence of degradation of the insoluble structures of the cell by leucocidin.

To detect a change in the lipids of the leucocyte the gross composition of the lipid fraction and the chromatographic behaviour of the phospholipids were investigated. Liberation of water-soluble constituents from the lipids should have been followed by their removal in the cell supernatant or the upper phase produced in washing the chloroform–methanol extract. Changes other than the liberation of water-soluble components should have been reflected in changes in the gross composition or chromatographic behaviour. The chloroform solution derived from the chloroform–methanol extract from 10^8 leucocytes contained 1·38 mg. of solids, 0·96 μmole of P, 1·8 μequiv. of esterified fatty acid, 0·14 μequiv. of total aldehyde, 0·002 μequiv. of free aldehyde, 0·31 μequiv. of amino N, 0·8 μmole of total N, 0·47 μmole of cholesterol. No differences could be detected in the composition of extracts of normal and leucocidin-treated leucocytes. Fig. 2 shows the separation curves for the phospholipids of normal leucocytes chromatographed on silicic acid, and five main bands can be distinguished. These were bulked as shown in Fig. 2. Between bands *A* and *B*, bands *C* and *D* and bands *D* and *E* respectively, the P content of the effluent did not fall to zero and the points of separation of the bands were taken as the fraction with the lowest P content. Plasmalogen and amino N were found in band *B* only; choline was present in bands *D* and *E* only; Band *B* is clearly heterogeneous, but as the degree of overlap varied in different experiments no assessment could be made of the relative proportions of the constituents.

Except for variations that could be ascribed to inadequate separation of the constituents of band *B*, no differences could be detected in the chromatographic behaviour of extracts from normal and leucocidin-treated leucocytes. In an experiment, in which extracts of normal and leucocidin-treated leucocytes were chromatographed on columns prepared identically, bands *A*–*E* contained 4·5, 28, 9, 28 and 24 % respectively of the phospholipid P from normal leucocytes, and 3·7, 31, 11, 25 and 23 % respectively of the phospholipid P of leucocidin-treated leucocytes.

Effect of leucocidin on the respiratory mechanisms of the leucocyte and macrophage. The inhibition of the ability of leucocytes to reduce certain respiratory dyes is the basis of the Neisser & Wechsberg (1900) method for detecting leucocidin activity. Consequently the possibility that leucocidin exerted a direct effect on the respiratory mechanisms of the cell was investigated. Table 2 shows that after treatment with leucocidin there is a decrease in the amount of lactic acid produced in whole leucocytes and macrophages but that there is no effect of leucocidin on lactate production in homogenates of the leucocyte.

Lactate production by whole rabbit leucocytes or macrophages was not affected by addition of only one of the two components of leucocidin, and lactate production by whole guinea-pig macrophages was not affected by the addition of both components of leucocidin.

It is known that macrophages can live for several days under anaerobic conditions (Harris & Barclay, 1955) and, similarly, glycolysis alone can provide the source of energy for many of the vital functions of the leucocyte (Elsbach & Schwartz, 1959; Sbarra & Karnovsky, 1959). It was therefore improbable that leucocidin exerted its toxic effect through inhibition of O_2 uptake, and in both leuco-

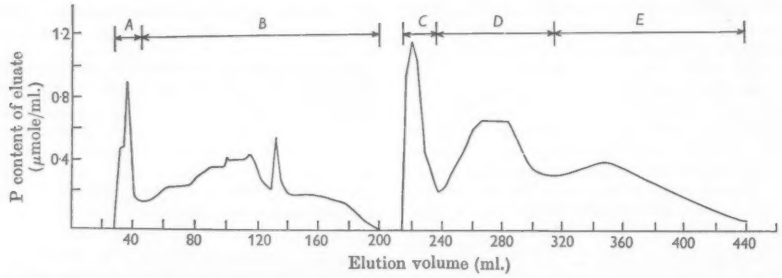


Fig. 2. Fractionation of the phospholipids of the leucocyte. An amount of lipid containing 75 μmoles of P was added to a 10 g. column of silicic acid. Fractions (2·2 ml.) were collected in the elution range 0–240 ml. and 5·5 ml. fractions in the elution range 240–500 ml. The elution volumes are measured after changing the eluent from chloroform to chloroform–methanol (9:1, v/v). At 200 ml. of elution volume, the eluent was changed to chloroform–methanol (2:3, v/v). *A*, *B*, *C*, *D* and *E* are bulked fractions.

cytes and macrophages there was no decrease in O_2 uptake till 15–20 min. after addition of leucocidin.

Permeability changes induced by leucocidin in leucocytes and macrophages. Table 3 shows that on incubation with leucocidin the cell swells, loses K^+ and gains Na^+ ions. No differences in water, K^+ or Na^+ ion content have been found in cells incubated with leucocidin for 5–20 min. These observations were made on centrifuged cell pellets. Elsbach & Schwartz (1959) showed that in the presence of fluoride and KCN leucocytes swell to the same extent as that found here for leucocidin-treated leucocytes. They measured the extracellular volume in centrifuged cell pellets similar to those prepared here and obtained values of 18% for normal cells and 40% for swollen cells. (The increased extracellular volume of the swollen cells probably results from their decreased density and less close packing during centrifuging.) Calculations based on these values give the K^+ ion concentration in μequiv./ml. of cell water to be 102 in normal cells and 19 in leucocidin-treated cells and establish that the changes in the K^+ ion content of the cell pellets cannot result from a changed extracellular volume. No changes in water or electrolyte content were observed when leucocytes or macrophages were incubated with either the *F* or the *S* component of leucocidin alone. The two components of leucocidin mixed had no effect on the water and electrolyte content of guinea-pig macrophages.

Table 4 shows that while about 75% of the K^+ ions of the cell is lost by leucocidin-treated leucocytes there is only a slight loss of amino acids (estimated as ninhydrin-reacting material), nucleotides (estimated as acid-soluble, ultraviolet-absorbing material) or P. No qualitative differences could be detected by paper chromatography of the

Table 2. *Effect of leucocidin on lactate production by leucocytes and macrophages*

Cells were incubated, alone or with added leucocidin, for 15 min. in glucose-free Hanks medium. For glycolysis in whole cells glucose and KCN were then added and incubation was continued for 30 min. For glycolysis in homogenates, cells were homogenized after the preliminary incubation and added to a mixture of substrates and cofactors, and incubation was continued for 30 min.

Glycolysis system	Lactate produced (μmoles/hr./10^8 cells)
Normal leucocytes	4·4
Leucocidin-treated leucocytes	1·1
Homogenate from normal leucocytes	6·3
Homogenate from leucocidin-treated leucocytes	6·2
Homogenate from leucocidin-treated leucocytes with added leucocidin	6·8
Normal macrophages	5·7
Leucocidin-treated macrophages	2·6

Table 3. *Effect of leucocidin on the water and electrolyte content of the leucocyte and macrophage*

The leucocytes were suspended in Krebs–Ringer phosphate solution and the macrophages in Hanks medium. The cells were incubated for 5 min., alone or with added leucocidin, and centrifuged, and the cell pellets analysed.

Cell	Water content (% of wet wt.)	K^+ ion content (μequiv./ml.)	K^+ ion content (μequiv./g.)	Na^+ ion content (μequiv./ml.)	Na^+ ion content (μequiv./g.)
Normal leucocyte	81	85	435	112	575
Leucocidin-treated leucocyte	92	14	155	175	1950
Normal macrophage	80	74	350	110	510
Leucocidin-treated macrophage	88	18	210	160	1800

Table 4. *Retention of some acid-soluble compounds by leucocidin-treated leucocytes*

Data are calculated from a series of similar experiments. The retention in the leucocidin-treated cell (column 2) is the amount in the pellet of leucocidin-treated cells expressed as percentage of the amount in the pellet from the normal cells. The distribution between the supernatant and leucocidin-treated cell (column 3) is the ratio concn. in pellet from leucocidin-treated cells/concn. in supernatant from leucocidin-treated cells.

Material	Retention in leucocidin-treated cell	Distribution between supernatant and leucocidin-treated cell
Potassium	25	1·65
Ninhydrin-reacting material	82	10·0
Ultraviolet adsorption at 260 mμ in N-HCl	85	16·5
Total nitrogen	80	13·0
Orcinol-reacting material	78	30·0
Phosphorus	68	6·5
Hexose monophosphate	95	—
Hexose diphosphate	100	—

Table 5. *Effect of leucocidin on the distribution of some phosphorus compounds in leucocyte suspensions*

After incubation in phosphate-free Krebs–Ringer bicarbonate the cell supernatants and centrifuged pellets were cooled in ice and treated with ice-cold trichloroacetic acid.

Cell fraction	Phosphorus content (μmole/10^8 cells)			
	Inorganic P	Creatine phosphate	Acid-labile phosphate	Total organic P
Normal-cell supernatant	0·028	0	0	0
Leucocidin-treated-cell supernatant	0·25	0·01	0·02	0·05
Normal-cell pellet	0·062	0·076	0·25	0·81
Leucocidin-treated-cell pellet	0·165	0·085	0·05	0·405

ninhydrin-reacting material in the supernatants or cell pellets of normal and leucocidin-treated leucocytes. In contrast 80 % of the P found in the supernatant of leucocidin-treated leucocytes is inorganic and thus differs from the P retained in the cell pellet.

Accumulation of inorganic phosphorus in the leucocidin-treated leucocyte. Table 1 shows that there is no net increase in the acid-soluble P of the leucocyte treated with leucocidin. Also, there is a release of inorganic but only little organically-bound P into the supernatant of leucocidin-treated leucocytes (Table 5). The analysis of the effect of leucocidin on the composition of the acid-soluble P compounds of the leucocyte was therefore extended and it was found that in leucocyte suspensions treated with leucocidin there is an accumulation of inorganic P (0·3–0·37 μmole/10^8 cells) and a corresponding decrease in the acid-soluble organically-bound P. The inorganic P and creatinine phosphate contents of normal leucocyte suspensions in phosphate-free Krebs–Ringer bicarbonate did not change during incubation for 30 min. at 37°.

The accumulation of inorganic P in suspensions of leucocidin-treated leucocytes was not prevented by addition of NaF (final concn. 0·2 M) or NaF and KCN (final concn. 0·2 M and 2 mM respectively) before addition of leucocidin. These substances did not prevent the liberation of the usual amount of protein into the supernatant of leucocidin-treated leucocytes (see below). The addition of adenosine triphosphate (4 mg./ml.) to the leucocytes subsequently incubated with leucocidin did not increase the accumulation of inorganic P. Leucocytes treated with streptolysin O, or disintegrated ultrasonically and then incubated in Hanks medium for 10 min., also liberated a similar amount of inorganic P, but in both these cases the effect was abolished by addition of fluoride. There was no accumulation of inorganic P in leucocytes incubated with iodoacetate (1 mM) or iodoacetate and cyanide (1 mM and 2 mM respectively).

Table 6 shows an analysis of some of the organic P compounds of the leucocyte and shows that there is a decrease in the adenosine triphosphate content of the leucocyte after leucocidin action. Hydrolysis to adenosine monophosphate could account for the accumulation of 50–60 % of the inorganic P. Hydrolysis to adenosine would account for nearly all the inorganic P accumulation.

Table 6. *Effect of leucocidin on the composition of the acid-soluble phosphorus compounds of the leucocyte*

	μmole/10^8 cells present in	
	Normal cells	Leucocidin-treated cells
Hexose diphosphate	0·010	0·010
Hexose monophosphate	0·005	0·005
Adenosine diphosphate	0·010	0·050
Adenosine triphosphate	0·100	0·009
Acid-labile P	0·24	0·095

Liberation of protein from the leucocyte treated with leucocidin. There was a considerable quantity of non-diffusible material in the supernatant of the leucocidin-treated leucocyte. The N content of this material was 14·5 %, indicating that little carbohydrate was present; the P content (0·014 %) indicated absence of significant amounts of phospholipid or nucleic acid, and no cholesterol, esterified fatty acid or unesterified fatty acid could be detected. On electrophoresis at pH 7·5 five components could be distinguished.

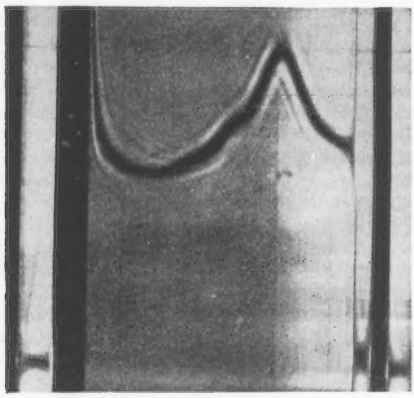
Table 7 shows that the release of protein from the leucocyte is not paralleled by a decrease in the soluble protein content of the soluble fraction of the homogenate. Similar results were obtained whether the homogenization was carried out ultrasonically or in a Potter–Elvehjem homogenizer and whether the homogenization medium was Hanks medium or 0·3 M-sucrose. Table 7 also shows that only a small amount of protein is found in the supernatant of leucocidin-treated macrophages. The same amount of protein was liberated from leucocytes treated with leucocidin in the presence of dextran (10 %, w/v), which prevented the cell swelling. Protein was also liberated from leucocytes treated with streptolysin O, but in this case there was a corresponding decrease in the protein content of the soluble fraction of the homogenate.

Fig. 3 shows that the soluble fraction of the homogenate from normal and leucocidin-treated

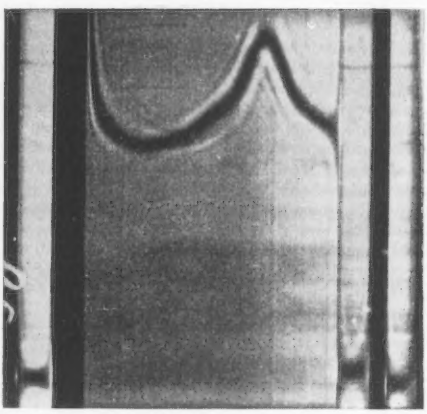
Table 7. *Effect of leucocidin on the protein distribution of leucocytes and macrophages*

After incubation of the cells alone or with added leucocidin, in Hanks medium, the supernatants were removed and the centrifuged cells homogenized in Hanks medium. The soluble fraction is the supernatant from centrifuging of the homogenate at 110 000*g* for 45 min.

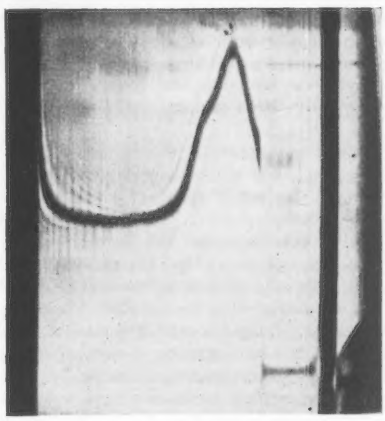
Cell	Protein content (mg./10^8 cells)		
	Cell supernatant	Soluble fraction, homogenized cells	Insoluble fraction, homogenized cells
Normal leucocytes	0·15	1·95	4·7
Leucocidin-treated leucocytes	1·5	1·85	3·5
Normal macrophages	0·03	3·0	—
Leucocidin-treated macrophages	0·3	2·9	—



(*a*)



(*b*)



(*c*)

Fig. 3. Ultracentrifuging of the soluble protein derived from normal and leucocidin-treated leucocytes. (*a*) Soluble fraction of homogenized normal leucocytes, 60 min. after reaching 59 780 rev./min. (*b*) Soluble fraction of homogenized leucocidin-treated leucocytes, 53 min. after reaching 59 780 rev./min. (*c*) Supernatant of leucocidin-treated leucocytes, 47 min. after reaching 59 780 rev./min.

Table 8. *Distribution of some antibacterial substances in fractions derived from normal and leucocidin-treated leucocytes*

Lysozyme contents are from comparison of activity with dilutions of egg-white lysozyme. Phagocytin units are the dilutions at the end points.

Cell fraction	Lysozyme content (μg./10^8 cells)		Phagocytin (units/10^3 cells)	
	Normal cell	Leucocidin-treated cell	Normal cell	Leucocidin-treated cell
Cell supernatant	1·0	125	<2	150
Sucrose-soluble fraction of homogenate	110	110	<2	<2
Citric acid extract of sucrose-insoluble fraction	20	20	300	200

leucocytes have very similar behaviour in the ultracentrifuge, and are distinct from the proteins in the supernatant of leucocidin-treated leucocytes. The main peak of the soluble fraction of the homogenized cells sediments about twice as fast as the protein in the leucocidin-treated cell supernatant.

Presence of antibacterial substances in the proteins liberated by leucocidin from the leucocyte. Hirsch (1960) showed that lysozyme is present in the soluble fraction of homogenized leucocytes. Phagocytin was confined to the insoluble fraction of homogenized leucocytes but could be extracted from the insoluble fraction by dilute citric acid. Table 8 shows that both lysozyme and phagocytin are present in the supernatant of the leucocidin-treated leucocyte. Table 8 also shows that the appearance of lysozyme in the supernatant of the leucocidin-treated cell is not paralleled by a decrease in the lysozyme content of the soluble fraction of the homogenized cell. It appeared therefore that the distribution of lysozyme in the leucocyte was bimodal, and that this is the case has recently been shown by Cohn & Hirsch (1960).

Readsorption of the proteins liberated from the leucocyte by leucocidin. It was possible that the protein in the supernatant of leucocidin-treated leucocytes was derived from a hypothetical macromolecular substance in the cytoplasm or cell surface. Table 7 shows that the protein present in normal cells but absent from leucocidin-treated cells is associated with the insoluble fraction of the homogenate. Incubation of the washed insoluble fraction of the homogenate of normal leucocytes with the two components of leucocidin (1 mg. of each component/ml. in Hanks medium) does not give rise to soluble protein. Also, when the protein in the supernatant of leucocidin-treated cells was added to the insoluble fraction of the homogenate it was readsorbed. Thus if leucocidin-treated leucocytes are homogenized in the presence of the cell supernatant then the total amount of soluble protein found in the homogenate is identical with that from homogenized normal cells.

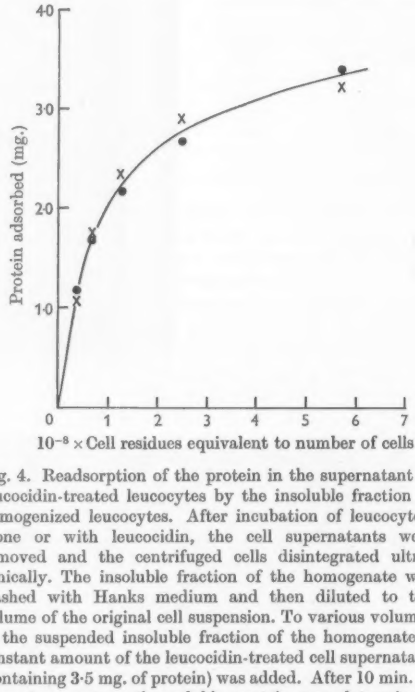


Fig. 4. Readsorption of the protein in the supernatant of leucocidin-treated leucocytes by the insoluble fraction of homogenized leucocytes. After incubation of leucocytes, alone or with leucocidin, the cell supernatants were removed and the centrifuged cells disintegrated ultrasonically. The insoluble fraction of the homogenate was washed with Hanks medium and then diluted to the volume of the original cell suspension. To various volumes of the suspended insoluble fraction of the homogenate a constant amount of the leucocidin-treated cell supernatant (containing 3·5 mg. of protein) was added. After 10 min. at room temperature the soluble protein was determined. ×, Adsorption by residues of normal cells; ●, adsorption by residues of leucocidin-treated cells.

Fig. 4 shows that the insoluble residues from 10^8 homogenized normal, or leucocidin-treated, cells readsorb the same amount of protein, about 2 mg. This is 30% greater than the amount released from normal leucocytes by leucocidin (1·5 mg./10^8 cells, Table 7).

DISCUSSION

It has been found that 10^5 molecules of each component of leucocidin are adsorbed by a leucocyte. The conditions of the adsorption, removal of 80% of the leucocidin from 9 ml. of solution by cells occupying about 2 ml., are such that the adsorption is not to be explained by pinocytosis (Lewis, 1931) but must result from adsorption on the cell surface.

The leucocidin-treated leucocyte rapidly loses most of its potassium but only small amounts of amino acids, nucleotides and phosphate esters. The slightly higher concentration of potassium in the leucocidin-treated cell pellet than that in the supernatant can result from about 5% of the cells being insensitive to leucocidin. The small amounts of amino acids and nucleotides that are released from the leucocyte by leucocidin may result not from increased permeability but from mechanical lysis of a small percentage of the leucocidin-treated leucocytes during the manipulations and centrifuging.

The release of potassium from the leucocidin-treated leucocyte could result from an increased permeability or from an interference with active transport. If the latter were the case then the loss of potassium in the leucocidin-treated leucocyte would be a measure of the rate at which potassium leaks from the normal cell and, as the potassium concentration in the normal cell is constant, of the rate at which it is normally accumulated. The minimum rate at which potassium is lost from the leucocidin-treated leucocyte is 960 μequiv./hr./ml. of cell water. Elsbach & Schwartz (1959) have measured the rate at which potassium is reaccumulated in leucocytes incubated at 37° after exposure to 0°, and from Fig. 2 of their paper it can be calculated to be about 20 μequiv./hr./ml. of cell water. It is therefore improbable that leucocidin acts by interference with the mechanism of active transport.

A change in permeability implies a structural change to the cell surface. This could conceivably be produced by mere combination of the two components with the cell surface. However, no permeability change accompanies adsorption of only one component of leucocidin, and the probability that the two components have an enzymic nature is made more plausible by the following considerations. There is an accumulation of inorganic phosphorus and a decrease in adenosine triphosphate in the leucocidin-treated leucocyte. Because of the failure of fluoride to inhibit this accumulation of inorganic phosphorus it is unlikely that it results from phosphatase action after cellular disorganization. That the partial respiratory failure in the leucocidin-treated leucocyte is not responsible for the accumulation of the inorganic phosphorus is shown by the failure of leucocytes poisoned with iodoacetate or cyanide to show a similar effect. It is possible therefore that the two components of leucocidin, adsorbed on the cell surface, set up a chain of reactions one stage of which requires adenosine triphosphate.

This paper has shown that the soluble protein appearing in the supernatant of the leucocidin-treated leucocyte is not derived from the soluble protein of the cell. It is unlikely that it is derived from the cell surface, for the quantity of protein involved is large and yet the change in permeability of the cell surface is very small. There is indirect evidence that the protein released from the leucocyte by leucocidin is derived from the granules. Gladstone & van Heyningen (1957) have shown that the granules of the leucocyte treated with leucocidin disappear in the first few minutes. Cohn & Hirsch (1960) have recently isolated the granules of the leucocyte and showed that they contain protein and phospholipid but only traces of nucleic acid. Lysozyme and phagocytin activities were found in the protein fraction. These two activities have been found in the proteins released from the leucocyte by leucocidin. Consistent with these proteins' having a granular origin is the fact that little protein is released from macrophages, which lack the characteristic granules of the leucocyte. Hirsch & Cohn (1960) showed that leucocytes lose their granules under a variety of conditions but that there is not a corresponding release of protein into the cell supernatant. As the leucocidin-treated leucocyte retains much of its normal impermeability to small molecules, the release of protein from the leucocyte suggests that the reactions set up by leucocidin have analogies with those involved in the secretion of protein.

SUMMARY

1. The *S* component and the *F* component of leucocidin are both adsorbed by the leucocyte from solutions containing either or both components.

2. Treatment of the leucocyte with leucocidin does not decrease the amount of nucleic acid sedimented by high-speed centrifuging of homogenized leucocytes.

3. The lipids of the leucocyte that are extractable with chloroform–methanol have been analysed and the phospholipids partially separated on silicic acid. The composition and chromatographic behaviour of the lipids is not changed by pretreatment of the cells with leucocidin.

4. Treatment with leucocidin decreases the rate of glycolysis in leucocytes and macrophages but leucocidin has no action on glycolysis in homogenates.

5. After treatment with leucocidin, leucocytes and macrophages swell, lose potassium and gain sodium. The permeability to amino acids, nucleotides and phosphate esters is only slightly changed. There is a release of inorganic phosphorus from the leucocidin-treated leucocyte.

6. There is an accumulation of inorganic phosphorus in the leucocidin-treated leucocyte and a corresponding decrease in the acid-soluble organic phosphorus content. There is a decrease in the adenosine triphosphate content. These effects are not inhibited by fluoride.

7. Protein is released from the leucocidin-treated leucocyte but is not derived from the soluble protein of the cell. Lysozyme and phagocytin activities have been found in the protein released by leucocidin.

8. The protein released by leucocidin from the leucocyte can be readsorbed by the insoluble fraction of homogenized leucocytes.

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The Metabolism of Exogenous *N*-Acetyl-D-glucosamine 6-*O*[^{35}S]-Sulphate in the Normal Rat

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Several workers have observed the excretion of inorganic and ester [^{35}S]sulphate after the administration of chemically synthesized polysaccharide [^{35}S]sulphates to laboratory animals (Morrow *et al.* 1952; Huseman, Hoffman, Lotterle & Widersheim, 1952; Ricketts, Walton & Saddington, 1954). Similar observations were made after the administration of ^{35}S-labelled biosynthetic preparations of chondroitin sulphate to rat (Dohlman, 1956; Dziewiatkowski, 1956) and of heparin to dog (Danishefsky & Eiber, 1959). None of the polysaccharide sulphates used so far is

suitable for specific examination of the ester sulphate group *in vivo*. The structures of both chemically synthesized and biosynthetic preparations are still incompletely defined, and in no instance have the positions of the ester sulphate groups on the carbohydrate moieties been established with certainty. Further, from the methods of preparation reported, the samples of chondroitin sulphate administered by Dohlman (1956) and Dziewiatkowski (1956) almost certainly consist of a mixture of the two isomeric forms, chondroitin sulphates A and C. Available evidence (summarized by Meyer, 1960) suggests that the sulphate group occupies position 4 of the *N*-acetyl-D-galactosamine residues in the A isomer, but is esterified to position 6 of these units in the C isomer. In α-heparin, as used by Danishefsky & Eiber (1959), the structure proposed by Wolfrom, Montgomery, Karabinos & Rathgeb (1950) suggests polysulphation of the polysaccharide chain with sulphate esterified to the amino group and position 4 of the D-glucosamine residues and to position 2 or 3 of some of the D-glucuronic acid moieties. Obviously, the use in metabolic studies of materials possessing [^{35}S]-sulphate groups esterified to several chemically different positions makes subsequent interpretation of experimental data excessively complicated.

Previous work (Lloyd, 1959*a*, 1960) has been concerned with the development of methods for the preparation of monosaccharide sulphates of defined constitution suitable for metabolic examination. The present paper describes observations on the metabolism of potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate in the rat. A preliminary account of part of this work has already been given (Lloyd, 1961).

MATERIALS AND METHODS

Potassium N-*acetyl*-D-*glucosamine* 6-O[^{35}S]-*sulphate*. This was prepared by a modification of the methods described by Lloyd (1959*a*, 1960). *N*-Acetyl-D-glucosamine (1·0 g.; Roseman & Ludowieg, 1954) was dissolved in 9 ml. of dry pyridine over 200 mg. of Drierite (L. Light and Co. Ltd.) at 50°, and the whole cooled to 5° before the dropwise addition of a mixture of 0·22 ml. of chloro[^{35}S]sulphonic acid (total activity 21 mc) and 0·13 ml. of unlabelled chlorosulphonic acid in 0·9 ml. of dry $CHCl_3$. After the mixture had been stirred at 5° for 30 min. its temperature was raised to 20° and stirring continued for a further 2 hr. On standing two layers were formed; the upper pyridine layer was removed by decantation and the lower oily layer was dissolved in water with vigorous stirring. Removal of inorganic [^{35}S]sulphate ions, pyridine and $CHCl_3$ was achieved as described by Lloyd (1960). The clear aqueous solution containing the crude [^{35}S]sulphate ester as the barium salt was concentrated to small volume, and the ester precipitated by the addition of 5 vol. of ethanol. Precipitated material was separated by centrifuging, and then recrystallized three times via the brucine salt before conversion into the potassium salt (Lloyd, 1960).

The homogeneity of the crude product was examined by electrophoresis on Whatman no. 1 filter paper in 0·1 M-ammonium acetate–acetic acid buffer (pH 4·0) for 2 hr. at 400 v. The strip was dried, sprayed with Morgan–Elson reagents (Partridge, 1948) and scanned for radioactivity. Three components were detected, two of which were radioactive and identical in electrophoretic mobility with authentic *N*-acetyl-D-glucosamine 6-*O*-sulphate and *N*-acetyl-D-glucosamine disulphate respectively. The third component was not radioactive and corresponded to *N*-acetyl-D-glucosamine (cf. Lloyd, 1959*a*, 1960).

The *N*-acetyl-D-glucosamine mono[^{35}S]sulphate was separated from the contaminating di[^{35}S]sulphate and parent compounds by zone electrophoresis on a cellulose column (80 cm. × 2 cm.) in 0·1 M-ammonium acetate–acetic acid buffer (pH 4·0) at 500 v. After 28 hr. the electrophoresis was stopped and the column eluted at 12 ml./hr. Fractions were monitored for radioactivity and where positive were examined by paper electrophoresis as above. Fractions containing a single component identical in electrophoretic mobility with the authentic 6-*O*-sulphate ester were pooled and concentrated to dryness by rotary evaporation at 35° followed by freeze-drying over KOH. The residue was dissolved in the minimum amount of water, adjusted to pH 7·6 with 0·05 N-KOH and precipitated by the addition of 10 vol. of ethanol. Precipitated material was separated by centrifuging, washed by suspension in ethanol (five times) and ether (three times) with intermediate centrifuging, and finally dried *in vacuo* over $CaCl_2$. The yield of dry product was 488 mg. The radioactivity of the product immediately after preparation corresponded to $2{\cdot}1 \times 10^4$ counts/min./mg. when measured after hydrolysis as an 'infinitely thick' plate of $Ba^{35}SO_4$ (see below).

Filter-paper electrophoresis showed the material to be homogeneous, identical in mobility with the authentic 6-*O*-sulphate ester and free from inorganic [^{35}S]sulphate. Descending chromatography on Whatman no. 1 filter paper, with butan-1-ol–acetic acid–water (50:12:25, by vol.) or *iso*butyric acid–aq. 0·5 N-NH_3 soln. (5:3, v/v) as solvent, showed only a single component, identical in chromatographic mobility with the authentic 6-*O*-sulphate ester.

Experimental animals. Male and female M.R.C. hooded rats (aged 2–3 months) maintained on a standard diet were used throughout. Injections were performed under light ether anaesthesia. In preliminary experiments animals were housed in the funnel type of metabolism cage, but in later experiments animals were placed in restraining cages modified from the design of Peacock & Harris (1950) in which urine and faeces could be collected strictly separately. The animals were allowed water without restriction and were removed from the cages for 30 min. in each 24 hr. when they were able to feed. Collected urines were filtered through a glass-wool plug, and the filtrate and washings used immediately or stored at −12°. Faeces were dried at 50° *in vacuo* before use.

Preparation of samples for the determination of radioactivity. The inorganic [^{35}S]sulphate fraction of rat urine was precipitated as follows. The collected urine and washings as above were diluted to 100 ml. A portion of the diluted urine (15 ml.) was transferred to a modified 50 ml. centrifuge tube (Walkenstein & Knebel, 1957) and 3 ml. of aq. 0·15 M-K_2SO_4 added. To the mixture was added 5 ml. of

4 N-HCl followed by 4 ml. of aq. 10% (w/v) $BaCl_2$. After careful mixing the tube was capped with Parafilm and kept at 2° for 6 hr. The $Ba^{35}SO_4$ precipitate was separated by centrifuging and washed three times by suspending in 40 ml. portions of water with intermediate centrifuging. After washing with 40 ml. of acetone and recentrifuging, the precipitate was dried at 110°.

For the determination of the combined inorganic and ester [^{35}S]sulphate fraction a sample (15 ml.) of the diluted urine was mixed with 5 ml. of 4 N-HCl and heated at 100° for 4 hr. After cooling, $Ba^{35}SO_4$ was precipitated as above. Preliminary experiments with these methods showed that (*a*) there was no co-precipitation of the ^{35}S-labelled ester in the presence of inorganic sulphate in rat urine, (*b*) the ester was not hydrolysed during the precipitation of the inorganic sulphate fraction, and (*c*) inorganic [^{35}S]sulphate could be recovered quantitatively in the presence of the labelled ester.

For the determination of the total ^{35}S content of urine a sample (15 ml.) was evaporated to dryness, the residue oxidized with fuming HNO_3 according to Young, Edson & McCarter (1949), and $Ba^{35}SO_4$ then precipitated as above. The same procedure was used in the determination of the total ^{35}S content of faeces or the whole carcass after digestion of solid with hot fuming HNO_3.

Determination of radioactivity. Precipitated $Ba^{35}SO_4$ was plated at 'infinite thickness' in plastic pans giving a surface area of 1 cm.^{2} The $Ba^{35}SO_4$ layer was then compressed with firm pressure with an accurately machined stainless-steel plunger. Measurements of radioactivity were made with a thin (1·4 mg./cm.^{2}) mica end-window Geiger–Müller tube in automatic scaling equipment (Nuclear-Chicago Corp., Ill., U.S.A.). Corrections were made for background, decay and coincidence, and sufficient counts were recorded to give a standard error of less than 2%.

Detection of radioactivity on paper chromatograms and paper electrophoresis strips. When material of sufficient activity was available components were detected on chromatograms and electrophoresis strips with a thin (1·4 mg./cm.^{2}) mica end-window Geiger–Müller tube in the C. 100 Actigraph chromatogram strip-scanner (Nuclear-Chicago Corp.). Alternatively radioautographs were obtained from chromatograms and electrophoresis strips kept in contact with Ilford Industrial B X-ray film for periods up to 21 days.

RESULTS

Rats were injected intraperitoneally with doses of 10 mg. or 20 mg. of potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate in 1·0 ml. of water. In initial experiments urine and faeces were collected at 24 hr. intervals to a total of 96 hr. However, the amount of total [^{35}S]sulphur appearing in the urine after 48 hr. was negligible (see Table 1) and in subsequent experiments urine and faeces were collected at 12 hr. intervals for 48 hr. only. After 48 hr. animals were killed by a blow on the back of the head and the carcass was used for the determination of residual ^{35}S. The results of these experiments are recorded in Table 2. Total ^{35}S values for faeces did not exceed 0·45% of the injected dose during the 48 hr. period and are not detailed.

Dohlman (1956) showed that organisms in the faecal flora of the rat were able to hydrolyse chondroitin [^{35}S]sulphate *in vitro*, 26% of the available radioactivity appearing as inorganic [^{35}S]sulphate overnight at 38°. The ability of faecal organisms of rat to hydrolyse *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate was tested in the following way. A solution of the ester (10 ml. of 0·2 M-sodium acetate–acetic acid, pH 6·2, containing 2 mg. of the ester/ml.) was mixed with an equal volume of a 2% (w/v) homogenate of freshly voided rat faeces, and the whole incubated at 21° or 37°. Samples (2 ml.) were withdrawn at various time intervals up to 48 hr., and diluted to 15 ml. with water. Solid material was removed by centrifuging, inorganic [^{35}S]sulphate precipitated from the clear supernatant as $Ba^{35}SO_4$, and the radioactivity determined as described. Control determinations were made in which a solution of the ester was incubated for the same time and at the same temperature, but to which the incubated faeces suspension was added only immediately before precipitating the inorganic sulphate fraction. Over 48 hr. inorganic [^{35}S]sulphate precipitated from the test incubated suspensions at 21° and 37° corresponded to 18 and 39% respectively of the total ester [^{35}S]sulphate available for hydrolysis (Fig. 1). When the ester was incubated at 37° for 36 hr. with a 2% (w/v) faeces suspension in 0·2 M-sodium acetate–acetic acid in the pH range 5·0–7·2 and inorganic [^{35}S]sulphate determined as above, a pH–activity curve with a maximum at pH 6·2 was obtained (Fig. 2).

Table 1. *Excretion of total urinary* ^{35}S *after the administration of potassium N-acetyl-*D*-glucosamine* 6-O[^{35}S]*-sulphate*

Results are expressed as a percentage of the injected dose. Mean values are given with ranges in parentheses. The radioactivity of the doses corresponded to $1·1 \times 10^5$ counts/min. (10 mg.) and $2·2 \times 10^5$ counts/min. (20 mg.) measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Sex	Dose (mg.)	No. of animals	Body wt. range (g.)	Total urinary ^{35}S 24 hr.	48 hr.	72 hr.	96 hr.
Male	10	3	117–152	89·4 (87·3–92·1)	2·1 (0·7–2·5)	0·7 (0·6–0·8)	0·4 (0·3–0·6)
Male	20	3	120–132	87·0 (85·6–89·5)	3·1 (2·8–3·3)	0·8 (0·7–0·9)	0·6 (0·5–0·8)

Table 2. *Distribution of radioactivity in rat urine and carcass after injection of N-acetyl-D-glucosamine 6-O[^{35}S]-sulphate*

Results are expressed as percentage of the injected dose. Mean values are given with ranges in parentheses. The radioactivity of the doses corresponded to $1{\cdot}1 \times 10^5$ counts/min. (10 mg.) and $2{\cdot}2 \times 10^5$ counts/min. (20 mg.) measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Sex	Dose (mg.)	No. of animals	Body wt. range (g.)	Distribution of ^{35}S in urine						Residual ^{35}S in carcass after 48 hr.
				Inorganic sulphate fraction			Combined inorganic and ester sulphate fraction			
				12 hr.	24 hr.	36 hr.	48 hr.	12 hr.	24 hr.	36 hr.	48 hr.	
Male	10	4	112–140	4·6 (3·2–5·7)	2·1 (1·2–2·4)	1·3 (0·6–1·8)	0·6 (0·2–0·9)	82·4 (78·9–88·4)	2·7 (2·1–3·2)	1·6 (1·4–1·9)	1·1 (0·9–1·2)	3·6 (2·4–5·2)
Male	20	4	150–180	3·5 (2·1–4·2)	1·9 (1·8–2·1)	1·2 (0·9–1·4)	0·5 (0·3–0·8)	84·8 (80·5–89·4)	2·4 (2·2–2·6)	1·5 (1·2–1·8)	0·9 (0·7–1·1)	5·2 (4·1–7·4)
Female	20	4	131–167	4·2 (3·8–5·3)	2·7 (1·9–3·2)	1·9 (1·5–2·7)	0·8 (0·6–1·1)	85·1 (79·4–88·6)	3·1 (2·1–3·7)	2·7 (1·9–3·2)	1·2 (0·8–1·6)	6·1 (5·9–7·2)
Male*	20	3	127–148	2·23 (1·4–3·1)	0·74 (0·5–1·1)	0·31 (0·2–0·4)	0·30 (0·17–0·38)	88·3 (84·2–91·1)	1·8 (1·6–2·3)	0·56 (0·48–0·64)	0·42 (0·36–0·49)	6·8 (5·1–7·9)
Male†	20	3	130–159	2·52 (1·9–2·8)	0·87 (0·7–1·4)	0·45 (0·3–0·6)	0·37 (0·28–0·49)	89·1 (85·4–93·6)	2·1 (1·8–2·5)	0·82 (0·72–0·91)	0·64 (0·52–0·73)	5·9 (4·9–7·2)

* Animals housed in restraining cages for collection of excreta.
† Animals pretreated with a total of 300 mg. of Terramycin and 1·5 g. of Sulphasuxidine by mouth during 70 hr. before injection and then housed in restraining cages for the collection of excreta.

In view of these findings urinary inorganic [^{35}S]sulphate observed in the initial studies *in vivo* could have arisen from the degradation of excreted ^{35}S-labelled esters by faecal organisms contaminating the urine during collection in the funnel type of metabolism cage. Consequently, a further series of experiments were performed *in vivo* in which injected animals were kept in restraining cages allowing the separate collection of urine and faeces. Results are recorded in Table 2.

Fig. 1. Liberation of inorganic [^{35}S]sulphate from potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate by the faecal flora of the rat. □, Incubated at 37°; △, incubated at 21°; ○, incubated at 37° with faeces from rats pretreated by mouth for 70 hr. with Sulphasuxidine and Terramycin (see text).

Fig. 2. Effect of pH on the liberation of inorganic [^{35}S]-sulphate from potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate by the faecal flora of the rat.

Alternatively, urinary inorganic [^{35}S]sulphate could have arisen after the excretion of ester [^{35}S]-sulphate compounds into the intestinal tract via the bile, followed by degradation by the intestinal flora and subsequent reabsorption of inorganic [^{35}S]sulphate from the large intestine (cf. Dohlman, 1956). In this context, Dodgson, Powell & Tudball (1960) have reported an appreciable reduction in the number of viable intestinal organisms when rats are treated by mouth with 300 mg. of Terramycin (Pfizer Ltd.) and 1·5 g. of Sulphasuxidine (Merck, Sharp and Dohme Ltd.) over a period of 70 hr. The effect of such treatment on organisms involved in the hydrolysis of *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate *in vitro* at 37° by a 2% (w/v) suspension of faeces obtained after the 70 hr. period is shown in Fig. 1. Animals pretreated according to the method of Dodgson *et al.* (1960) were injected with the labelled ester and housed in restraining cages for the collection of excreta. The results of these experiments are recorded in Table 2.

Analysis of urine by paper chromatography and paper electrophoresis. Samples of urine collected at 12 hr. and 24 hr. intervals after the injection of 20 mg. of potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate were freeze-dried and the residue was dissolved in the minimum amount of water. Portions (2 μl.) of the resulting solution were examined by electrophoresis on Whatman no. 1 filter paper in 0·1 M-ammonium acetate–acetic acid buffer (pH 4·0) at 400 v for periods of 2–4 hr. The paper strips were dried and scanned for radioactivity. The same qualitative pattern was apparent in all samples examined. A faster-moving component of low activity corresponded to inorganic [^{35}S]sulphate, and a slower-moving band accounted for the greater part of the radioactivity applied to the strip. Scanner diagrams and radioautographs of 12–24 hr. urines revealed the same pattern as the 0–12 hr. urines, but of very much lower intensity. In no instance were electrophoretically-immobile components observed, suggesting the absence of neutral ^{35}S-labelled compounds from the urines collected at both time intervals.

For paper chromatography, samples of urine as above were freeze-dried, redissolved in the minimum amount of water and then dialysed twice against 100 ml. portions of water for 4 hr. on a shaking dialyser. Preliminary experiment showed that 98–99% of the total ^{35}S content of 12-hr. urines was dialysable during this treatment. The diffusates were combined and after they had been concentrated to small volume inorganic [^{35}S]-sulphate was precipitated by the cautious addition of 5 mM-$BaCl_2$. The mixture was clarified by centrifuging and the clear supernatant concentrated to small volume by freeze-drying. Portions (2 μl.) of the concentrated supernatant were examined by downward chromatography on Whatman no. 1 filter paper with butan-1-ol–acetic acid–water (50:12:25, by vol.) as solvent for periods up to 72 hr. The chromatograms were then dried and scanned for radioactivity. In each case chromatography revealed that the major constituent of the urines was identical in mobility with authentic *N*-acetyl-D-glucosamine 6-*O*-sulphate. By computing the area under the curve in scanner diagrams this component was found to account for an average of 97% (range 95–98%) of the treated 12 hr. urine applied to the chromatogram. Examination of radioautographs of the same chromatograms revealed the presence of three further components with mobilities relative to the 6-*O*-sulphate ester of 0·35, 0·74 and 1·25 respectively. However, from the scanner diagrams each of these components corresponded to only 1% or less of the radioactivity originally applied to the chromatogram. Scanner diagrams and radioautographs of treated 12–24 hr. urines revealed the same pattern, but once again of very much lower intensity.

DISCUSSION

When potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate was administered intraperitoneally to rats, between 82 and 89% of the radioactivity was excreted during the first 12 hr. after the injection. This rate of excretion is higher than that observed after the administration of polysaccharides containing hexosamine [^{35}S]sulphate residues (Dohlman, 1956; Dziewiatkowski, 1956; Danishefsky & Eiber, 1959). However, it has been shown that the excretion of radioactivity in the urine after the administration of xylan [^{35}S]-sulphate was related to the average chain length of the preparation and that the higher the degree of polymerization the lower was the rate of excretion (Huseman *et al.* 1952). There can be little doubt that the relatively low molecular weight of the ester used in the present study contributes to the observed high rate of excretion.

Although the greater part of the radioactivity excreted during the 48 hr. after injection consisted of *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate, significant quantities of inorganic [^{35}S]sulphate also appeared in the urine during this period. When excreta were collected in the funnel type of metabolism cage a total of 7·1–9·6% of the radioactivity excreted in 48 hr. corresponded to inorganic [^{35}S]sulphate. However, when restraining cages were used and cross-contamination of urine and faeces could not have occurred, the inorganic [^{35}S]sulphate fraction was reduced to 3·5–4·3% in 48 hr. This suggests that inorganic [^{35}S]sulphate observed in initial experiments could have arisen

at least in part from metabolism of the excreted ester by faecal organisms contaminating the urine during collection. On the other hand, depression of the gut flora has little effect on the amount of inorganic [^{35}S]sulphate excreted, suggesting that the intestinal organisms are unlikely to play a major part in the metabolism of the ester *in vivo*. The observed desulphation of the ester by organisms from the intestinal tract is of interest in that liberation of sulphate from monosaccharide sulphate esters by bacterial enzymes has been reported previously only by Tanko (1932).

The consistent appearance of 3·5–4·3% of the injected dose as inorganic [^{35}S]sulphate provides additional evidence that mammalian tissues are in some way able to separate the ester sulphate bond of hexosamine sulphate esters (cf. Dohlman, 1956; Dziewiatkowski, 1956; Danishefsky & Eiber, 1959). Of the possible routes that exist it appears unlikely that a specific carbohydrate sulphatase is involved in this process. Dodgson & Lloyd (1961) tested a wide variety of tissue preparations from rat, pig and human against the 6-*O*-sulphate esters of D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. In no instance could the liberation of inorganic sulphate be detected with either the chloranilate (Lloyd, 1959*b*) or benzidine (Dodgson & Spencer, 1953) micromethods, although each of these esters is known to function as a substrate for the glycosulphatase of *Littorina littorea* (Lloyd, 1959*c*; Dodgson, 1961; Dodgson & Lloyd, 1961). Alternatively, inorganic sulphate might appear as a result of the complete degradation of the *N*-acetyl-D-glucosamine sulphate molecule. Lutwak-Mann (1941) examined the degradation of hexosamines and their *N*-acetyl derivatives by mammalian tissues and recorded the appearance of ammonia and unknown compounds. Since it is proposed (see Roseman, 1959) that the first step in the degradation of D-glucosamine and *N*-acetyl-D-glucosamine is the formation of the corresponding 6-*O*-phosphate ester it is improbable that *N*-acetyl-D-glucosamine 6-*O*-sulphate can be metabolized by this route. Finally, in the presence of an appropriate sulphokinase the sulphate group of *N*-acetyl-D-glucosamine 6-*O*-sulphate might be transferred to adenosine 3′:5′-diphosphate to give adenosine 3′-phosphate 5′-sulphatophosphate. Subsequently, inorganic sulphate might appear directly from the breakdown of the sulphatophosphate or after the transfer of the sulphate group from it to yield a sulphate ester labile in the presence of mammalian tissues. Precedents exist for each of these possibilities (see Gregory & Robbins, 1960).

The earlier synthetic (Lloyd, 1959*a*, 1960) and infrared (Lloyd & Dodgson, 1959, 1961) studies, on hexose and *N*-acetylhexosamine sulphate esters of defined constitution in relation to the structure of naturally occurring polysaccharide sulphates (Lloyd, Dodgson, Price & Rose, 1961), and the present metabolic study assume new significance in the light of observations on the structure of keratosulphate. This sulphated polymer, reported as a constituent of cornea (Meyer, Linker, Davidson & Weissmann, 1953), cartilage (Meyer, Hoffman & Linker, 1958) and nucleus pulposus (Gardell, 1955), has been shown to contain *N*-acetyl-D-glucosamine, D-galactose and ester sulphate together with 1–2% of a methyl pentose (Rosen, Hoffman & Meyer, 1960). Results of methylation studies (Hirano, Hoffman & Meyer, 1960) suggest that the main structure of keratosulphate contains the repeating unit β-D-galactopyranosyl-*O*-(*N*-acetyl-β-D-glucosamine 6-*O*-sulphate). After the injection of *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate into rats 3·6–6·8% of the radioactivity is retained in the carcass after 48 hr. However, because inorganic [^{35}S]sulphate is released from the ester *in vivo* it is not possible to decide whether this retention is due to a direct incorporation of the intact radioactive ester into the keratosulphate fractions of rat or to the general incorporation of inorganic [^{35}S]sulphate into all the sulphate esters of rat tissues.

SUMMARY

1. Potassium *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate was administered intraperitoneally to male and female rats. The main feature of the metabolism of the ester was the rapid elimination of radioactivity, 82–89% of the dose appearing in the urine in the first 12 hr.

2. Although the greater part of the radioactivity was excreted as ester sulphate, inorganic [^{35}S]-sulphate also appeared in the urine in the 48 hr. period after injection.

3. Organisms from the faecal flora of rat were shown to metabolize the labelled ester *in vitro*, with the liberation of inorganic [^{35}S]sulphate, but available evidence suggests that the intestinal flora are not primarily responsible for the metabolism of the ester observed *in vivo*.

4. Paper-chromatographic examination of 12 hr. and 24 hr. urine samples showed that the radioactivity associated with the ester sulphate fraction was due largely to unchanged *N*-acetyl-D-glucosamine 6-*O*[^{35}S]-sulphate; three additional compounds of unknown structure and low radioactivity were also detected.

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The Isolation of the α-Toxin of *Clostridium welchii* Type A by Zone Electrophoresis in Vertical Columns

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The α-toxin of *Clostridium welchii* type A is believed to be the most important single factor involved in clostridial toxaemia (van Heyningen, 1950, p. 25). It has been extensively studied biochemically and serologically, as a result of which it has been recognized as a lecithinase capable of splitting phosphorylcholine from lecithin (Macfarlane & Knight, 1941). Such enzymic activity is believed sufficient (Macfarlane, 1950) to explain the haemolytic, necrotizing and lethal activities assigned to it by Glenny, Barr, Llewellyn-Jones, Dalling & Ross (1933). Previous attempts have been made by van Heyningen (1941*a*, *b*), Adams & Hendee (1945), van Heyningen & Bidwell (1948) and Roth & Pillemer (1953) to isolate it from the other *C. welchii* antigens such as hyaluronidase, collagenase and θ-toxin (a reversibly oxidizable haemolysin), with which it usually occurs in culture filtrates. The techniques employed by these workers were mainly fractional precipitation with salt and organic solvents.

A series of experiments was planned in which the newer techniques applicable to such a problem were to be applied, and the first attempt was by zone electrophoresis in vertical columns. At the conclusion of the experimental stage of this part of the work, a paper was published by Habermann (1959), who described the results of a similar approach to the same problem. He found that the α-toxin could be purified to a considerable degree by a combination of either fractional precipitation and chromatographic techniques or electrophoretic and chromatographic techniques. The yield from the chromatographic stage was high but that from

the electrophoretic stage was relatively poor, and the α-toxin was not quantitatively resolved from θ-toxin by the latter.

MATERIALS AND METHODS

Organisms. Clostridium welchii strain CN.1491 was used throughout this work and was kindly given by the Wellcome Research Laboratories. Stock cultures were kept in Robertson's alkaline egg medium (Levine & Schoenlein, 1930). Fresh cultures were prepared about every 3 months and stored at 4°.

Culture media

Cooked-meat broth. Ox muscle was substituted for heart in the preparation described by Mackie & McCartney (1948), otherwise the medium was the same.

Medium for production of α-toxin. Peptone (Evans Medical Supplies Ltd.) and Bacto-Casitone (Difco Laboratories Inc., Detroit, Mich., U.S.A.) were each added to 3 l. of tap water to a final concentration of 1% (w/v). The pH was adjusted to 8 and the solution steamed for 30 min., after which it was filtered. N-Hydrochloric acid was then added to bring the pH to 7·8. Meat, with superficial fat removed, was minced, treated with 0·05N-NaOH (1 l./kg. of minced meat) for 20 min. at just below boiling point and squeezed dry. The meat particles (200 g./l.) were added to the peptone–Casitone solution and the whole was autoclaved at 15 lb./in.² for 15 min. An aqueous solution of glucose (50%, w/v) was autoclaved at 15 lb./in.² for 15 min. and, immediately before inoculation with the culture, was added to the medium to a final concentration of 0·6% (v/v).

Medium for production of θ-toxin. The medium used was that described by Brooks, Sterne & Warrack (1957).

Enzyme and toxin assays

α-Toxin. α-Toxin was assayed as lecithinase by a method based on that described by van Heyningen (1941*a*), in which the turbidity produced in egg-yolk solutions is measured. This technique is not completely specific, other enzymes being known which will evoke the same phenomenon. However, it is assumed (cf. Macfarlane & Knight, 1941; Kushner, 1957) that if this reaction is specifically neutralized by appropriate antisera it can be used for the assay of the lecithinase produced by *C. welchii* type A. This condition was fulfilled with preparation A (see Results section). A standard toxin was prepared by dialysing a culture filtrate against glycerol for 72 hr. (Macfarlane & Knight, 1941) and examined by Mrs I. Batty of the Wellcome Research Laboratories and found to contain 14·95 lecithovitellin (LV) units/ml. (1 LV unit is that amount of α-toxin which in the presence of 1 i.u. of antitoxin will just cause turbidity in an egg-yolk solution: see van Heyningen, 1950, p. 27); this preparation remained stable for 2½ years. The egg-yolk broth was made by suspending 10% (v/v) broken yolks of eggs in papain-digest broth which included peptone 1% (w/v) and NaCl 0·5% (w/v). After centrifuging this was Seitz-filtered. The composition of the borate buffer diluent was as given by Howie, Duncan & Mackie (1953), but the pH was adjusted to 7·4 instead of 8·0–8·4.

Volumes of 5 ml. of the egg-yolk broth in borate buffer (15%, v/v) were dispensed into standard EEL (Evans Electroselenium Ltd., Harlow, Essex) nephelometer tubes. Dilutions of preparation A, in the range 0·09–0·33 LV unit/ml., were made in steps of 0·03 LV unit/ml. and 1·0 ml. was added to each assay tube. The tube contents were mixed rapidly by inversion and placed at 1 min. intervals in a water bath at 37° and incubated for 15 min., after which they were withdrawn at 1 min. intervals, wiped dry and the turbidity was read on the nephelometer. Each assay was done in duplicate and the results were used to construct a standard curve. This procedure was repeated before each assay because of the variability of the egg-yolk solutions.

Proteolytic activity. Proteolytic activity of our preparations was assayed with azocoll as substrate (Oakley, Warrack & van Heyningen, 1946). The method of assay was based on that described by Bidwell (1950). Azocoll was prepared according to Oakley *et al.* (1946) and sieved through an 80-mesh sieve. The diluent used throughout was NaCl, 0·85%, to which was added tricresol ether, 0·5%.

The test system was calibrated in terms of arbitrary units; preparation A, which was rich in 'azocoll-splitting' activity, was used as standard and said to contain 1·0 unit/0·0135 mg.

Solutions containing from 1 to 10 azocoll units/ml. were made and 1 ml. portions were pipetted into EEL tubes (8 mm. diam., suitable for EEL colorimeter), each of which contained 20 mg. of azocoll, and incubated overnight at 37° after mixing. Residual azocoll was centrifuged and the extinction of the supernatant measured in the colorimeter. One batch of azocoll was sufficient for the whole of this work and did not require repeated standardization.

Hyaluronidase. Hyaluronidase was assayed by the turbidimetric assay described by Dorfmann & Ott (1948) with serum albumin (Armour Laboratories, fraction V).

θ-Toxin. The reversibly oxidizable haemolysin (θ-toxin) was assayed as an antigen against specific antibody. The procedure of Brooks *et al.* (1957) was followed.

Glycosidases. α-Mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase and β-glucuronidase were assayed by the methods described by Conchie, Findlay & Levvy (1959).

Ultrafiltration

The high-pressure ultrafilter described by Bullen & Batty (1956) was used for the concentration of our culture filtrates. Membranes were cast from 8% collodion in acetic acid (Necol; Imperial Chemical Industries Ltd.). Full details of membrane production and the manipulation of the apparatus are given by Bullen, Thurlbourn & Brown (1954).

Small volumes, e.g. eluate fractions, were conveniently concentrated by the 'polyethylene glycol' technique described by Kohn (1959).

Zone electrophoresis in columns

The apparatus was a development of the one described by Mould & Stephen (1958). Attempts to cool packed columns centrally in order to increase the applied field have been made by introducing a cold-finger type of arrangement (Porath, 1957). The disadvantage of such a design is in the local evolution of heat at the porous disk at the bottom of the column.

The following is a description of the construction of an annulus column (i.e. one in which the refrigerant goes right

through the porous disk). The essential part of the apparatus is shown in Fig. 1.

A is a column, 65 cm. long and 4 cm. diam., with a B34 standard ground-glass socket at the top and a B34 standard ground-glass cone at the bottom. A central tube *B*, 1 cm. diam., emerges from the side of the column just below the socket at the top and terminates at the lower end in a B10 socket *C*, flush with the lower edge of the B34 cone. The porous disk *D* is formed *in situ* from an Araldite resin and a 'filler' (all materials supplied by Aero Research, Duxford, Cambs.). The composition of the mixture was Araldite 103 (10 g.), Hardener 951 (1 g.) and Silica flour (15 g.). This was allowed to set overnight at room temperature, after which a series of holes was drilled in the plug. The anode chamber consists of a three-necked 5 l. vessel, into the bottom of which is sealed a tube *E* of 1 cm. diam., terminating in a B10 cone. This is designed so that, with the column *in situ*, the B10 cone of *E* engages the B10 socket of *B*. To obviate glass-blowing difficulties, the seal *F* in the bottom of the flask is made with the same Araldite mixture as for the plug. The rest of the apparatus was exactly as described by Mould & Stephen (1958). The effective field across the column is about 80% of the applied p.d.

Cellulose. Ethanolysed cellulose was prepared according to Flodin & Kupke (1956). Our experience showed that the best source of cellulose was Regaid cotton wool (Boots), although variations in the suitability of the ethanolysed product were detected from batch to batch.

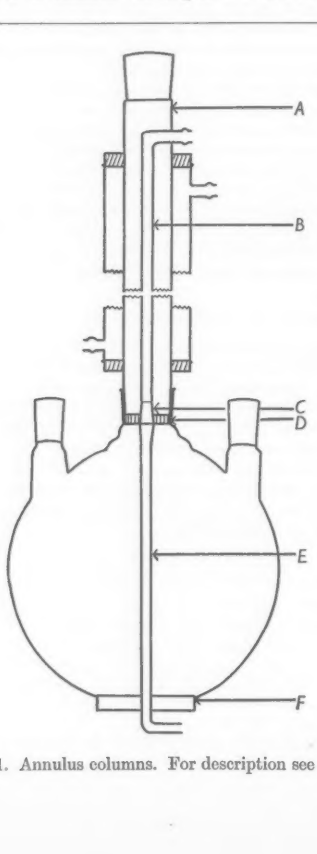


Fig. 1. Annulus columns. For description see text.

Buffers. Borate buffer, pH 7·1, *I* 0·025, which contained 0·025 M-NaCl, was used since at this pH the α-toxin is stable (Gale & van Heyningen, 1942) and in borate buffer can be conveniently assayed as lecithinase.

Packing columns. Ethanolysed cellulose, 200 g., was suspended in borate buffer, pH 7·1, *I* 0·025. The column was packed to a depth of 55 cm. by allowing this slurry to filter into the column at a controlled rate, from a height of 12 ft. A filter-paper disk had been previously placed on top of the porous disk, and one was finally placed on top of the packing to prevent its being disturbed. The suitability of the packed column was always tested by passing through it a sample of the material to be fractionated, the eluate being examined for activity recovery and any evidence of adsorption.

Immunoelectrophoresis

This was largely carried out according to Grabar & Williams (1953, 1955).

Glass plates, 30 cm. × 11 cm., were coated with New Zealand agar, 1% (w/v) in borate buffer, pH 7·1, *I* 0·05, to a depth of 3 mm. The agar was cut into a convenient number of strips and inlays were cut with a cork borer of suitable diameter. The positions of the inlays were determined by the electrophoretic properties of the substances under study, due allowance being made for the electro-endosmotic streaming through the agar gel. The sample was made up in buffer to twice the concentration finally desired and diluted with an equal volume of agar, 2% (w/v) in buffer. This was introduced into the inlay. Paper wicks connected the ends of the gels to the buffer in the electrode chambers.

The plates were usually clamped to a refrigerated copper plate whose temperature was maintained at 1°. A voltage gradient of 2·6 v/cm. was applied, which produced currents of approx. 46 mA.

The electrode chambers were perfused continuously with fresh buffer to maintain a stable pH with borate buffer, pH 7·1. At the end of the electrophoretic run, antiserum, previously dialysed against the same buffer, was added to the longitudinal ditches of the gel, which was then usually incubated for 48 hr. at 37°. The gels were then washed in a solution of NaN_3, 0·1%, and NaCl, 0·85%, for approx. 3 days with frequent changes of the wash solution. The precipitin lines were then stained with azocarmine G (G. T. Gurr Ltd., London, S.W. 6).

Other chemical and biological estimations

Nitrogen. The nitrogen content of specific samples was estimated by the micro-Kjeldahl technique.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate the protein in fractions from the columns. Standards were not prepared as only the relative distribution of protein in the fractions was examined. Readings were taken at λ 500 mμ.

Measurements of pH. All pH measurements were carried out with a Pye pH meter.

LD_{50} *Assays.* Mice (23–25 g.; T.O. strain, Mill Hill 1956) were used. The mice were injected intravenously with a total volume of 0·3 ml. of toxin dilution. Deaths were recorded at 24, 48 and 72 hr. Very few deaths were observed after 48 hr. and none after 72 hr., which was taken

as the maximum time limit for recording death from toxic effects. Adequate serum and diluent controls were included with the experimental groups.

Minimum haemolytic dose (MHD). The haemolytic potency of the lecithinase in preparation A was estimated as the number of MHD/LV unit. Doubling dilutions were made from a solution containing 1 LV unit/ml. in a saline diluent containing 5 mM-$CaCl_2$. A suspension of washed, sheep red blood cells, 6% in 0·85% NaCl soln., was made, 0·5 ml. portions being dispensed into each tube. The tubes were incubated for 1 hr. at 37°, and then cooled and centrifuged. The last tube to contain trace haemolysis was taken as the end point. (The content of θ-toxin in preparation A, produced in the presence of solid meat particles, was so low as to be negligible at this dilution.)

RESULTS

Production of α-toxin

Cooked-meat broth (20 ml.) was inoculated with 2 ml. of stock culture and incubated overnight at 37°. This culture was subcultured twice in cooked-meat broth (20 ml.) and once in the peptone medium (200 ml.) used for toxin production. Each subculture was grown for approx. 2 hr. (or until active gas production was observed) at 37°. The last subculture was used as inoculum for the 3 l. batch of peptone medium, which was then incubated for 3½ hr. at 37°. The culture was clarified through paper pulp and sterilized through a Carlson E.K. pad. The filtrate thus obtained contained 3·5 LV units/ml. and was concentrated by ultrafiltration.

Viable bacterial counts (Bullen & Scarisbrick, 1957) showed that most of the α-toxin was produced during active cell division, in confirmation of the earlier observation of Gale & van Heyningen (1942). Under normal conditions of culturing the pH dropped from an initial pH of 7·8 to about 6. Addition of bicarbonate buffer was shown to have no appreciable effect on the yield of α-toxin.

Concentration of Seitz filtrates

Potent crude material was obtained by high-pressure ultrafiltration with reasonable success. Filtrates from cultures grown in media containing peptone, 1% (w/v), could be concentrated almost to dryness. The viscous concentrate was washed off with a NaCl solution (0·1%), the washings being pooled, centrifuged if necessary until the supernatant was absolutely clear, and freeze-dried. Preparation A was the product obtained from four 3 l. batches of culture, the filtrates of which were separately concentrated and then pooled and freeze-dried. Preparation B was produced in a manner similar to that for preparation A, except that the culture was grown in a 3 l. batch of the medium described for θ-toxin production.

α-Toxin production was directly proportional to the peptone concentration of the medium over the range 1–3% (w/v). However, the culture filtrates from media containing peptone of initial concentration >1% (w/v) could not be concentrated to the same degree as those from media containing ⩽1% (w/v) peptone.

Zone electrophoresis

Preparation A (500 mg. equivalent to 500 LV units of α-toxin, 15 000 i.u. of hyaluronidase, 37 000 azocoll units of proteolytic activity) were dissolved in 2 ml. of the same buffer as was used for packing and run into the column to a depth of 3 cm. The column was then fitted into the rest of the apparatus, which was filled with buffer. A potential of 1000 v was applied, giving an approximate voltage gradient of 16 v/cm. across the packed column and producing a current of 78 mA. After 18 hr. the column was removed and eluted with the same buffer, 5 ml. fractions being collected. Each fraction was analysed for protein, α-toxin (as lecithinase), hyaluronidase and proteolytic activity. The result is shown in Fig. 2. The proteolytic activity appeared to be distributed between four components, and the hyaluronidase was recovered as a broad asymmetric peak. The α-toxin was recovered in 90–95% yield, the proteolytic activity in 95% and the hyaluronidase in 70–75% yield. The hyaluronidase and the proteolytic activities were clearly not separated. Immunoelectrophoresis showed the α-toxin to be contaminated with both faster- and slower-moving antigens.

A second experiment was done with preparation A, the duration of the run being 40 hr., and at the half-way stage the buffer was changed in the side

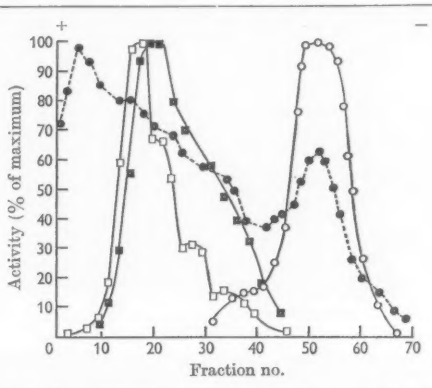


Fig. 2. Zone electrophoresis: 18 hr. run on preparation A, showing separation of α-toxin from the proteolytic and hyaluronidase activities. ○, α-Toxin; □, proteolytic activity; ■, hyaluronidase; ●, protein. Activities are plotted as percentages of their respective maxima.

arm and the cathode vessel. Under these conditions the hyaluronidase and proteolytic activities were run right off the column and the α-toxin was recovered in 85–90 % yield. Immunoelectrophoresis showed the isolated toxin to be a single precipitin system (see below). The results of the 18 and 40 hr. experiments show that the percentage recovery of the active toxin decreases with the duration of the experiment; this confirmed the observations of Habermann (1959). The material was not run again on the column as this would have resulted in a further substantial loss of activity.

Table 1. *Properties of α-toxin before and after fractionation*

Activity of the preparations is expressed in terms of lecithovitellin (LV) units, lethal dose (LD_{50}) and minimal haemolytic dose (MHD).

LV units/mg. of N	LD_{50}/LV unit	MHD/LV unit
Crude toxin (preparation A)		
7·8	55	16–32
Fractionated toxin		
50·6	55	16–32

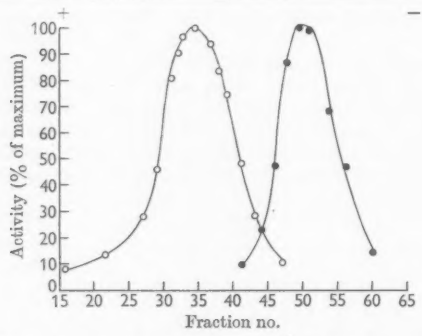


Fig. 3. Zone electrophoresis: 40 hr. run on preparation B, showing separation of α-toxin from θ-toxin. ○, α-Toxin; ●, θ-toxin. Activities are plotted as percentages of their respective maxima.

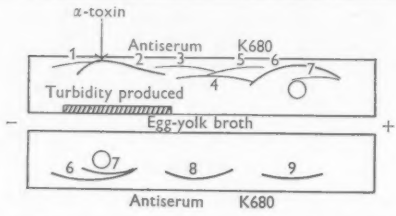


Fig. 4. Diagrammatic representation of the result obtained with preparation A with egg yolk and antiserum K680.

The properties of the isolated toxin are listed in Table 1.

An overall purification of approximately sixfold was achieved when expressed on a unit nitrogen basis. The lecithinase, lethal and haemolytic properties of α-toxin were not separated by electrophoresis.

A third experiment was done with preparation B to determine whether or not the α- and θ-toxins were electrophoretically separable. The equivalent of 500 LV units of α-toxin and 1300 units of θ-toxin were dissolved in 2 ml. of borate buffer and the experiment was conducted in exactly the same manner as described for the second experiment; the result is shown in Fig. 3. Under these conditions the α-toxin is more anodic than the θ-toxin and these two substances are clearly separable. The yield of θ-toxin was variable, 50–70 %, and, unaccountably, the yield of α-toxin was considerably lower, 50–60 %, from this preparation than that repeatedly obtained from preparation A. The fractions which contained the α- and θ-toxins respectively were shown by immunoelectrophoresis to be complex mixtures of antigens.

Immunoelectrophoresis

A borate gel with two strips was prepared. Two inlays were cut in the gel, one in each strip. One was near the cathode and one, in the other strip, was near the anode end, and into these preparation A (12 mg.) was introduced. At the end of the run (i.e. after 18 hr.) antiserum K680 (*Clostridium* Diagnostic Serum, *C. welchii* type A, produced by Wellcome Research Laboratories) was added to the two outer ditches and the egg-yolk solution used for lecithinase assays added to the centre ditch. After incubation nine precipitin lines developed in the gel, and an intense turbidity developed in the centre ditch which later spread into the gel itself. This was rather difficult to photograph and a diagrammatic representation is given in Fig. 4. The line opposite the zone of turbidity was assumed to be the one produced by the α-toxin and its corresponding antitoxin. This line is also indicated by an arrow in Plate 1 (*a*), which shows the result of repeating the experiment without the egg-yolk treatment. Another experiment was done with a third strip in the gel, in

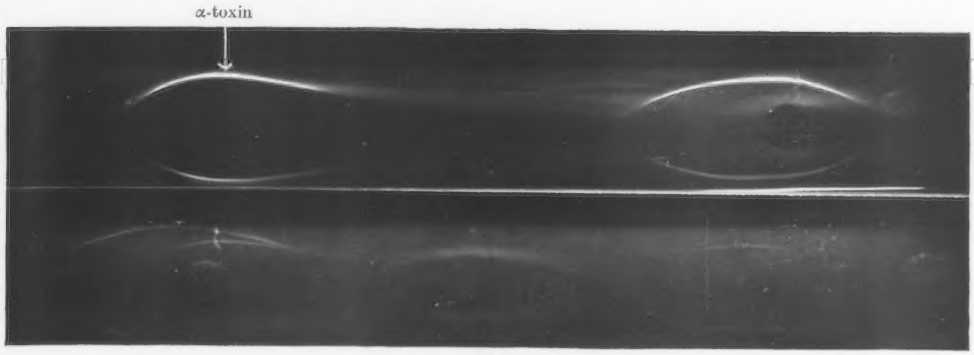
EXPLANATION OF PLATE 1

(*a*) Immunoelectrophoresis of preparation A. The precipitin line (marked with an arrow) is the one opposite which the egg-yolk turbidity developed.

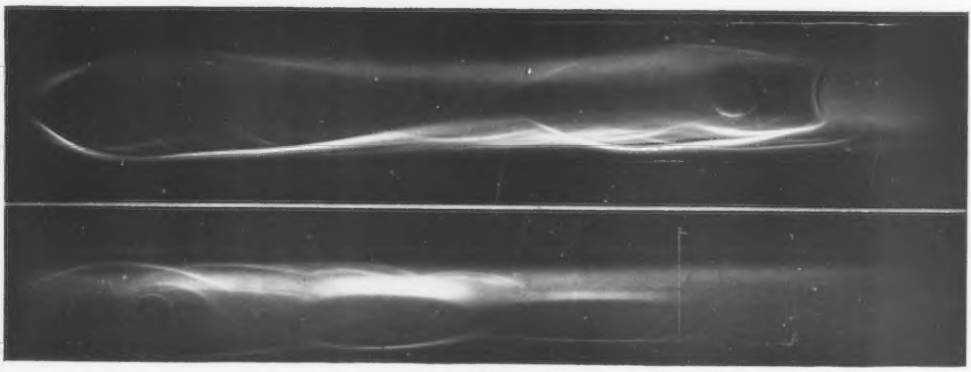
(*b*) Immunoelectrophoresis of preparation B.

(*c*) Immunoelectrophoresis of preparation A. Recovered on elution through a column packed with ethanolysed cellulose.

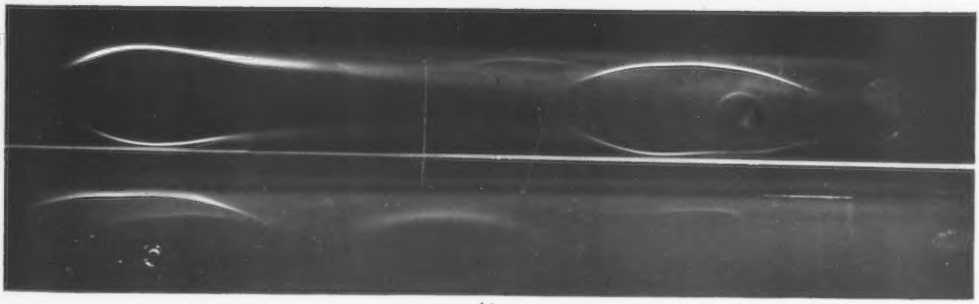
(*d*) Immunoelectrophoresis of isolated α-toxin.



(*a*)



(*b*)



(*c*)

(*d*)

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(*Facing p.* 582)

which was placed (near the anode end) the α-toxin isolated from the 40 hr. zone-electrophoresis run, previously described, with preparation A. A precipitin line appeared [Plate 1 (*d*)], whose position indicated a substance with the same mobility as that which produced the precipitin line shown by the arrow in Plate 1 (*a*); no other lines were observed. These experiments were repeated with 18 different *C. welchii* antisera, some of which revealed the same number of lines in preparation A as did serum K 680, but none revealed more.

By the use of the neutral indicator *N*-2:4-dinitrophenylethanolamine it was shown that the α-toxin was anionic, migration towards the cathode being due to the considerable electroendosmosis which occurred through these agar gels. It was also demonstrated, by repeating the experiment with dinitrophenylethanolamine in a phosphate-buffered agar (pH 7·1, *I* 0·05) that the effect of boric acid on the mobility of proteins having a carbohydrate moiety (Garbers & Joubert, 1958) was not operative with the α-toxin.

Preparation B, originally prepared for θ-toxin, was shown to contain at least 25 antigens when examined against serum K 680 [Plate 1 (*b*)]. The abundance of precipitin lines produced by these preparations prompted the search for other specifically identifiable substances. Preparation A was examined for α-mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase and β-glucuronidase, of which the two last-named are usually associated with hyaluronidase and the first two with the second two in mammalian tissues. None of these enzymes was detected. Preliminary histochemical tests (Lillie, 1954) also failed to detect deoxyribonuclease, which is known to be produced by some *C. welchii* type A strains (Oakley & Warrack, 1953).

A buffer solution (2 ml.) containing 100 mg. of preparation A was then passed through a column, packed as for the electrophoresis runs but without application of the electric field. The eluate was concentrated by polyethylene glycol and examined immunoelectrophoretically; the result is shown in Plate 1 (*c*) and is seen to be similar to (*a*), obtained with the original untreated preparation A.

Both toxigenic media used for the production of preparations A and B were concentrated approximately tenfold and examined against serum K 680, no precipitin lines being developed. Preparation A was also tested against a serum from a rabbit immunized with the toxigenic peptone medium and again no result was obtained.

DISCUSSION

The method of concentration of the Seitz filtrates was regarded as an important aspect of this work, as the obtaining of crude concentrates rich in α-toxin has been cited (Roth & Pillemer, 1953) as one of the major hindrances in purification studies. The crude ultrafiltered concentrates could be obtained within 24 hr. of the start of culture operations without their being subjected to precipitation with salts or organic solvents; a stage yield of 95% was obtained.

It is possible to obtain the α-toxin of *C. welchii* type A free from hyaluronidase, θ-toxin and the proteolytic activities, which degrade the crude substrate azocoll, by zone electrophoresis.

The α-toxin isolated from a 40 hr. run with preparation A as starting material was shown by immunoelectrophoresis to have been separated from the other eight antigens of bacterial origin that were originally present. Nevertheless the degree of purification achieved was only sixfold. The activity of the final material is very low compared with that of the product obtained by Roth & Pillemer (1953), which although of undefined antigenic purity, was 35 times as active. Failure to demonstrate precipitin lines with concentrated culture medium against antiserum K 680 would suggest that the low specific activity of the isolated toxin was due to the presence of non-antigenic residues from the medium, which were retained by the ultrafiltration membranes used in the concentration of the culture filtrates. Alternatively, the low activity could mean that there were present bacterial antigens to which there were no antibodies present in the 18 *C. welchii* antisera examined, but in view of the extremely varied composition of these antisera this is regarded as unlikely.

Oakley & Warrack (1953) listed type A strains of *C. welchii* as being capable of producing at least five serologically distinct antigens. The results reported in this paper show that culture filtrates from peptone-based meat-containing media may contain at least nine different antigens and those from muscle-digest meatless media 25 different antigens.

Examination of crude material passed through the column without application of the electrical field showed that passage through the packed column and the method for concentrating the eluate did not alter the serological properties of the mixture studied. This was considered important in respect of the analysis of the isolated α-toxin as it shows that the processes involved in fractionating and recovering the active material do not result in the denaturation of antigenic contaminants.

SUMMARY

1. Culture filtrates of *Clostridium welchii* type A, concentrated by high-pressure ultrafiltration, have been examined by zone electrophoresis and immunoelectrophoresis.

2. Zone electrophoresis was carried out with ethanolysed cellulose as the stabilizing medium in columns of a novel design. The columns were internally as well as externally cooled; a description of their construction is given. With such an apparatus, the α-toxin of *C. welchii* type A was quantitatively separated from the hyaluronidase, proteolytic and θ-toxin activities. The lecithinase, haemolytic and lethal activities were electrophoretically inseparable.

3. Immunoelectrophoresis was carried out on the toxin preparations. At least nine different antigens were found in the one crude preparation and at least 25 in another. The α-toxin obtained from the former preparation by zone electrophoresis was shown to be free from the other eight antigens but was of low specific activity.

I wish to thank Dr J. J. Bullen, Head of Pathology Department, Rowett Research Institute, for his enthusiastic support; Dr D. L. Mould (now at Animal Diseases Research Association, Moredun Institute, Edinburgh) for initial guidance in the electrophoretic procedures and his stimulating interest throughout; Mr B. F. Fell of the Pathology Department of the Rowett Research Institute, for the deoxyribonuclease histochemical examination; Dr J. Conchie of the Enzymology Department of the Rowett Research Institute, for the α-mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase and β-glucuronidase examinations; Mrs I. Batty of the Wellcome Research Laboratories for the standard α-toxin assay; the Wellcome Research Laboratories for the gift of valuable antisera and culture CN. 1491; Miss Jean McBain, Mr W. G. Gordon and Mr G. M. Bruce for technical assistance, and Mr I. Robb and Mr R. Middleton for the photography.

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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

1. ENCEPHALOMYOCARDITIS VIRUS IN KREBS II MOUSE-ASCITES-TUMOUR CELLS

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Many attempts have been made during recent years to define the relationship between protein and nucleic acid biosynthesis. There is now adequate evidence that soluble proteins such as ribonuclease, insulin or haemoglobin have fully-defined chemical structures, each with a defined amino acid sequence. These sequences appear to be under genetic control and numerous hypotheses have been advanced to explain the control mechanism. In essence, they are all variants of one proposition, that protein synthesis takes place upon a template. Only three types of molecule have been considered as serious candidates for this role, deoxyribonucleic acid, ribonucleic acid and protein. Although deoxyribonucleic acid is closely associated with the genetic apparatus, current evidence favours ribonucleic acid as the macromolecule most closely associated with protein synthesis. There seems no doubt that ribonucleic acid can carry the necessary information for control of protein synthesis; thus, for example, ribonucleic acid from tobacco mosaic virus can induce the synthesis of strain-specific tobacco mosaic protein (Gierer & Schramm, 1956; Fraenkel-Conrat & Singer, 1957), and various ribonucleic acid preparations obtained from virus-infected mammalian cells or from mammalian viruses can induce synthesis of new virus (Colter, 1958). It seems quite likely therefore that ribonucleic acid could act as a template for protein synthesis, but no direct demonstration of this function has been achieved.

If a template exists, it is essential that a purified preparation of the template material should be isolated, and for this material to be recognized it is essential that it should have a measurable biological activity. It seemed possible that encephalomyocarditis virus grown in Krebs II mouse-ascites-tumour cells by the methods developed by Sanders (Sanders, 1957; Sanders, Huppert & Hoskins, 1958) might provide a system that would meet our requirements. The present series of papers represent studies designed to define the biological and biochemical characteristics of this system.

In particular, attention was directed to the effects of virus infection on the protein and nucleic acid metabolism of the host cell, with the object of determining the site within the cell and the time in the infectious cycle at which the protein and nucleic acid components of the virus are synthesized.

The advantages of the encephalomyocarditis–Krebs II cell system may be defined as follows. The host cell can be propagated on a large scale without technical complication and, moreover, the cell suspension can be maintained in a simple synthetic medium. The 'take' rate of virus in the host cell approaches 100% in a single growth cycle so that biochemical analyses of a cell population are unlikely to be vitiated by random variation throughout that population. In addition, the host cell contains an infective ribonucleic acid (template?), which may be isolated by phenol extraction (Huppert & Sanders, 1958), and the pure virus may be obtained as a crystalline ribonucleoprotein (Faulkner, Martin, Sved & Work, 1960). It is unlikely that a direct demonstration of the template function of ribonucleic acid could be achieved in intact cells, but the Krebs II cell gave good cell-free preparations capable of protein synthesis (Martin, Malec, Coote & Work, 1961). Since viral protein may be readily assayed or, if necessary, isolated by immunological or haemagglutination techniques, the system seems to offer the possibility of the direct induction of synthesis of a specific protein by a specific ribonucleic acid in a cell-free preparation.

The present paper describes the standard methods that have been used throughout these investigations for the propagation of Krebs II cells and for the growth of encephalomyocarditis virus in these cells. The effects of virus infection upon the nucleic acids and proteins of the host cell have been measured both by direct quantitative analyses and by measurement of turnover rate with [^{14}C]valine and [^{14}C]orotic acid as markers.

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METHODS

Buffers and nutrient solutions

Abbreviations. EMC, Encephalomyocarditis; PBS, phosphate-buffered saline.

The following solutions were used throughout the investigations reported in successive papers of this series.

Phosphate-buffered saline. Three stock solutions were prepared. Solution *A* contained NaCl (40 g.), KCl (1 g.), Na_2HPO_4 (5·75 g. or equivalent of hydrated salt), KH_2PO_4 (1·0 g.) and water (4 l.). Solution *B* contained $MgCl_2,6H_2O$ (0·5 g.) in water (500 ml.). Solution *C* contained $CaCl_2$ (0·5 g.) in water (500 ml.). Each solution was autoclaved and stored at 0°. For use solutions *A*, *B* and *C* were mixed in the proportions 40:5:5 and benzylpenicillin (100 i.u./ml.) and streptomycin–$CaCl_2$ (60 μg./ml.) (Glaxo Laboratories) were added. This solution is referred to throughout as PBS.

Earle's medium. Four stock solutions were prepared. Solution *A* contained NaCl (68 g.), KCl (4 g.), $MgSO_4,7H_2O$ (2 g.), phenol red (0·1 g.) and water (400 ml.). Solution *B* contained $NaH_2PO_4,2H_2O$ (1·58 g.), glucose (20 g.) and water (400 ml.). Solution *C* contained $CaCl_2$ (2·0 g.) in water (200 ml.). Solution *D* contained $NaHCO_3$ (4·4 g.) in water (100 ml.). These solutions were autoclaved and stored at 0°. For use solutions *A* (4·0 ml.), *B* (4·0 ml.) and *C* (2·0 ml.) were mixed and diluted to 100 ml. with sterile water; 5 ml. of this mixture was discarded and replaced by 5 ml. of solution *D* previously saturated with CO_2. Antibiotics were added as above. Where the Earle's medium was to be used for plaque assay the phenol red was omitted. This medium is similar to that of Earle (1943) and will be referred to as Earle's medium.

Phosphate-buffered gelatin. For assay of virus haemagglutinin, gelatin (0·2 g.; Difco Laboratories, Detroit, Mich., U.S.A.) was dissolved in boiling water (about 25 ml.), cooled, mixed with glucose (4·5 g.) and made up to 100 ml. with water. This solution was stored at 0°. It was mixed with an equal volume of PBS just before use.

Agar. Bacto-agar (150 g.; Difco Laboratories) was washed by decantation with water (20 times in all with 4 l. of water each time) and thrice with acetone (A.R. grade; 500 ml. for each wash). The agar was dried on open trays and dissolved in water as required (3 g. of agar in 100 ml. of water autoclaved for 10 min. at 10 lb./sq.in.).

Glassware. All glassware to be used in experiments on virus growth was washed by a method recommended by Dr F. K. Sanders (personal communication). This method was an essential requirement for reproducible results. Calgon (Albright and Wilson Ltd., 20 g.) was mixed with water (2270 ml.) and sodium metasilicate (180 g.) and, after 24 hr. the mixture was filtered. All glassware was cleaned in a neutral detergent (e.g. Lissapol; Imperial Chemical Industries Ltd.), rinsed and soaked overnight in the diluted silicate solution (1 part to 99 of water). The solution was heated to boiling and the glassware was removed, rinsed in tap water, soaked in 0·1 N-HCl (30 min.), rinsed in distilled water and steam-sterilized. Pipettes were cleaned in H_2SO_4–HNO_3 mixture and treated as above.

Krebs II mouse-ascites-tumour cells. The tumour-cell line used in this work originated as a mammary carcinoma and was converted into the ascites form by the method of Klein & Klein (1951). Dr J. Craigie, Imperial Cancer Research Institute, Mill Hill, kindly supplied mice bearing these Krebs II tumour cells and this cell line was used throughout. In order to obtain reproducible results it was necessary to propagate the cells in female mice. The strain of mouse was not critical and three strains of hybrid albino mice were used, Parkes, T.O. and Schneider. As judged by the ability to produce large yields of blood-free ascites fluid the Parkes strain was, however, preferable.

Production of a standard ascites-tumour-cell preparation. A cell line constantly propagated through mice might be subject to mutational change over a large number of successive generations and it was therefore considered advisable to maintain a stock of frozen cells as a standard preparation and to build up fresh experimental stock from these cells as required. In this way it has been possible throughout these investigations to use cells that were not more than five passages removed from the parent stock. Since, however, Krebs II cells are subject to temperature shock, some attention had to be paid to the conditions of preparation and storage of the standard cells.

Female Parkes mice aged 7–8 weeks and weighing 20–22 g. were used. This narrow range is specified because younger mice had a high mortality rate and older mice produced ascitic fluid containing few Krebs cells and large amounts of blood and fatty material. The mice were injected intraperitoneally with washed tumour cells (see later). About 1×10^7 washed cells gave a high yield of ascitic fluid in 7–8 days and a median survival time of 12 days. Smaller inocula produced solid infiltrative growths and bloody peritoneal exudates. After 7 days the ascitic fluid was withdrawn under sterile conditions. The usual yield of fluid was within the range 5–10 ml. and the cell count about 1×10^8/ml.

Aqueous glycerol helps to protect frozen tissues (Smith, 1954), and addition to the fresh ascitic fluid of glycerol to a concentration of 6% (v/v) increased the resistance of Krebs cells to freezing (12% of glycerol was less effective). A number of experiments were conducted to determine the optimum conditions for freezing. A suspension of cells plunged into ethanol–CO_2 at about −65°, then thawed and injected into mice, gave a 55% take rate (200 mice), whereas cells cooled during 5–7 min. to −70° showed a 65% take rate and cells cooled over 1 hr. a 75% take rate as compared with fresh cells. An apparatus was constructed (cf. Mathews, Maida & Buthala, 1959) that facilitated controlled cooling of cell suspensions. Ampoules of ascites-tumour-cell suspensions were prepared as follows. Ascitic fluid was collected from Parkes mice about 8 days after inoculation and all samples discoloured by blood were discarded. The pooled ascitic fluid was mixed thoroughly with glycerol (final concentration, 6%, v/v), dispensed into ampoules (4 ml.), flame sealed, and stored in a cold room at −4° for 1 hr. The ampoules were then transferred to a cold bath and the temperature was lowered 1·5°/min. for 10 min. to −15° and more slowly to −25° (15 min.). As soon as the ampoules had reached −25° they were cooled rapidly to −70° and maintained at this temperature.

When cells were required for the propagation of the tumour a single ampoule was thawed rapidly by agitation in water at 40° for about 2 min. Slow thawing (15 min.) gave a cell preparation with about one-quarter of the potency of rapidly thawed material. When cells were prepared, frozen and thawed as described above, 0·1 ml. of

cell suspension produced large ascitic tumours in 7 days in over 80% of the injected mice and the number of mice dying before 7 days was not more than 5%.

The first passage from the original tumour supplied by Dr Craigie was used to prepare a batch of the standard stock of cells. This stock was used either to replenish the standard stock or for the production of the large batches of ampouled material used to produce tumour for experimental purposes. Cells prepared in this way and stored at −70° have retained their viability unaltered for periods up to 8 months.

Preparation of Krebs II ascites-tumour cells for experimental use

Fresh ascitic fluid obtained as described above was transferred to 50 ml. sterile centrifuge tubes, diluted with about twice its volume of ice-cold PBS, and mixed at once. The cells were spun down at 400 rev./min. (60*g*) for 10 min. and the supernatant was discarded. Cells were rewashed at least twice in the same way or more often if necessary until free from erythrocytes and fat clumps. A sample of the cell suspension was diluted about 50-fold with 0·2% of nigrosin in PBS (water-soluble nigrosin, E. Gurr Ltd., London, S.W. 14) and the number of stained (dead) and unstained (live) cells counted in a Neubauer haemocytometer (Kaltenbach, Kaltenbach & Lyons, 1958). The number of dead cells did not exceed 10% and was usually below 5%. Nigrosin was found preferable to eosin (Sanders, 1957), since with the latter dye the proportion of dead cells rises rather rapidly with time as a result of the toxic action of the dye.

Growth of virus

Origin of virus. The strain of EMC virus used in these studies was obtained from Dr F. K. Sanders, M.R.C. Laboratories, Carshalton, Surrey. The virus was derived from the original strain isolated by Helvig & Schmidt (1945). A number of mice were injected intraperitoneally with the suspension of virus, and 36–40 hr. later those mice that were partially paralysed were killed and their brains removed under sterile conditions. The brains were homogenized in 0·9% NaCl, centrifuged and injected intraperitoneally into a second batch of mice. The virus was passaged in this way once more and an homogenate of two infected mouse brains was then added to a suspension of 1×10^8 Krebs II ascites-tumour cells in 5 ml. of Earle's medium and incubated under the standard conditions set out below. When the proportion of dead cells was 80% the cells were spun down and the supernatant was used to infect a further series of ascites-cell suspensions. The virus produced by this second cycle of growth in Krebs cells was harvested, ampouled and stored at −70°. This material provided the primary virus culture. Stocks of secondary cultures, built up from this primary culture, were used for experimental purposes.

Incubation conditions. Krebs II cells were washed in cold PBS as described above, transferred to Earle's medium, spun down and resuspended in fresh medium in a stoppered flask at a known cell concentration (2×10^7/ml.). A standard preparation of virus was added (3 plaque-forming units/host cell), and, after sufficient time to allow for adsorption of virus (30 min.), the flask was transferred to a mechanical swirler, which swirled the flask gently at about 70 rev./min. at 36°. The shape of the flask used probably was not critical; however, in the experiments described here, all incubations were performed in Erlenmeyer flasks containing one-tenth of their volume of cell suspension.

Assay of virus. The methods used were those developed by Sanders (1957). The plaque assay involved addition of 0·1 ml. of a suitable dilution of virus to a fresh preparation of washed ascites cells ($1{\cdot}2 \times 10^8$ cells/1·2 ml.) in Earle's medium without phenol red. The cells were then mixed with warm agar (1·8 ml. of 1·5%, w/v, in Earle's solution at 44°). The infected cells were layered upon a disk of preset agar (about 12 ml. of 1·5% agar in Earle's solution in a Petri dish), spread evenly and allowed to set. Plates were incubated for 2½ days at 37°, flooded with neutral red (0·0001% in 0·9% NaCl) and left for 1 hr. at 37°, the dye solution was discarded, and the plaques were counted. It was essential to maintain the plates, both during preparation and during incubation, in 5% CO_2, or alternatively to incubate in the presence of Pardee buffer as described by Bellett (1960). A stock virus preparation was always assayed along with each set of plaque assays.

As an alternative to plaque assay we have at times used cell-death titration (Sanders, 1957). In this method serial dilutions of virus were added to freshly washed Krebs II cells and incubated under the standard incubation conditions. After 15 hr. samples were withdrawn and mixed with nigrosin, and the percentage of dead cells was counted as already described. The relationship of this assay to the plaque is given by Sanders. The stock virus assayed by plaque count gave 3×10^9 plaque-forming units/ml. The same virus preparation assayed by cell-death titration gave 2×10^9 infective particles/ml. and also gave a haemagglutinin titre (see below) of 12 000–25 000.

Estimation of haemagglutinin. EMC virus agglutinates sheep red cells. Jungeblut (1958) has summarized earlier evidence that the haemagglutinating activity is part of and not just associated with the infective agent. This view is amply confirmed by our own studies on crystalline virus (Faulkner, Martin, Sved, Valentine & Work, 1961) since we have found that the ratio of haemagglutinin to infectivity remains constant throughout purification. The standard haemagglutination technique used throughout the present series of papers is given below.

Fresh sheep red cells (defibrinated sheep blood without preservative; Burroughs Wellcome Ltd., Beckenham) were washed in three changes of 0·9% NaCl and suspended in Alsever's solution (1 ml. of packed cells/10 ml. of solution as described by Bukantz, Rein & Kent, 1946). In this state cells could be stored at 3° for 7 days. When required, cells were washed in fresh 0·9% NaCl by three successive cycles of centrifuging (800*g* for 5 min.) and suspended in PBS–gelatin to a cell concentration of $2{\cdot}5 \times 10^7$ cells/ml. A suitable dilution of virus was prepared, also in PBS–gelatin (usually 1 in 100) and serial one-half dilutions of this stock solution (0·5 ml.) were made in standard M.R.C. agglutination plates; PBS–gelatin was used for serial dilution. A sample (0·25 ml.) of the red-cell preparation was added to each dilution of virus and the plates were left for 18 hr. at 4°. The end point was taken as the highest dilution of virus able to prevent formation of a complete ring of red cells. The titre, expressed as haemagglutinin units/ml., is defined as the reciprocal of this dilution. The stock virus had a titre of 12 000–25 000 haemagglutinin units/ml.

Rapid haemagglutinin method. At times, it was advantageous to have available a method of rapid haemagglutinin assay requiring minutes rather than hours. This was especially important in virus purification as it enabled several steps in the purification procedure to be carried out during 1 day (cf. Faulkner *et al.* 1961). The method adopted gave an assay in about 30 min. Serial dilutions of virus (1 ml.) were made in 15 ml. glass centrifuge tubes. The tubes were cooled in ice and 1 ml. of the standard sheep-red-cell preparation (also cooled at 0°) was added to each tube. After 15 min. at 0° the tubes were centrifuged for 3 min. at 1500*g* (swing-out buckets). The tubes were removed singly, held against a good light and agitated gently by slow swirling. In the absence of virus the cells swirled up evenly from the small hard button at the bottom of the tube. Starting at the highest dilution of virus, the first tube in which the cells failed to be resuspended evenly was taken as the end point (1/8000 with the stock virus). With progressively larger quantities of virus, the red cells became resuspended as progressively larger clots.

Extraction and estimation of nucleotides, nucleic acids and proteins

Preparation of cells. Much of the analytical work was carried out on Krebs II cells that had been incubated either with or without virus in Earle's medium. Before analysis these cells were washed as described below. The suspension of cells in the incubation flask was cooled to 0°, mixed with an equal volume of ice-cold PBS and transferred to capped centrifuge tubes. The flasks were rinsed with cold PBS, the washings mixed with the cells, and the whole was centrifuged at 120*g* for 5 min. at 0°. The supernatant was discarded. The cells were resuspended in cold PBS and the volume was made up to twice the original incubation volume. After centrifuging a second time the cells were rewashed in the same way, transferred to analytical homogenizer tubes (Kamphausen & Morton, 1956) and centrifuged at 2200*g* for 5 min. at 0°, and the cell pellet was stored at −20°.

Acid-soluble nucleotides. The cell pellet was thawed, homogenized with water (2 ml. for 2×10^7 cells) at 0°, mixed with cold M-$HClO_4$ (1 ml.) and rehomogenized. Insoluble material was collected (2200*g* for 5 min.) and washed first with 3 ml. and then with 2 ml. of 0·25M-$HClO_4$. The pooled acid extracts contained the acid-soluble nucleotides.

Removal of lipid. The acid-insoluble residue was washed with 5 ml. of methanol at 0° and the washings were discarded. After a second methanol wash at room temperature the residue was extracted with 4 ml. of methanol–$CHCl_3$ (1:1, v/v) for 30 min., then with 4 ml. of ether–$CHCl_3$ (1:1, v/v) for 30 min. and then washed twice with 4 ml. of ether. On each occasion the solids were homogenized with the solvent and removed by centrifuging.

Total nucleic acid. The lipid-free residue was homogenized with 3 ml. of 0·5M-$HClO_4$, heated at 70° for 30 min. and cooled to 0°. Cooling was necessary otherwise an $HClO_4$-soluble protein was precipitated at a later stage. The cold $HClO_4$ extract was separated by centrifuging and the residue re-extracted at 70° with $HClO_4$ (1 ml.) for 10 min. The residue from the second extraction was washed once with 0·5M-$HClO_4$ (1·0 ml.), and the combined acid extracts were pooled and diluted to 5·0 ml. This material is referred to as total nucleic acid.

Nucleic acid was estimated by extinction measurement at 264 mμ in $HClO_4$. For direct estimation of P the method of Griswold, Humoller & McIntyre (1951) was used and for conversion of extinction into μg. of P the relationship was $E_{1\,cm.}/0{\cdot}290 = \mu g.$ of nucleic acid P/ml. (cf. Ogur & Rosen, 1950).

Total protein. The residue from the nucleic acid extraction was homogenized with water (1 ml.) and 0·2N-NaOH (3·0 ml.) was added. The mixture was heated to 50° for 30 min. and centrifuged. The residue was re-extracted twice, first at 50° as above and then at room temperature. The insoluble residue was mainly powdered glass from the homogenizer but a small residue of alkali-insoluble protein was always obtained. The pooled alkaline extracts were diluted with 0·2N-NaOH as required and the extinction at 290 mμ was measured. The relationship between extinction and total protein N was determined after Kjeldahl digestion of a standard sample of tumour-cell protein, either by the standard distillation method or by use of Nessler's reagent as described by Lang (1958). If the amount of protein was too small for estimation by ultraviolet absorption, the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used. For calibration of the colour yield/μg. of N, a standard sample of tumour-cell protein was used. This standard protein was prepared by extracting a large batch of washed Krebs cells by the methods given above to remove acid-soluble material, lipid and nucleic acids. The residue was washed free of acid with methanol–water (90:10, v/v), dried and stored in a vacuum desiccator.

Separation of ribonucleic acid and deoxyribonucleic acid. The method was based on that of Davidson & Smellie (1952). The lipid-free cell pellet (see above) was homogenized with 10% (w/v) NaCl (10 ml.) buffered at pH 6·0 with sodium acetate (0·1 M) and heated at 90° with constant shaking for 12 hr. The extract was mixed with 95% ethanol (2·5 vol.) and left overnight at −20°. The supernatant from the ethanol precipitation was separated and discarded. It contained no measurable quantity of DNA and only small amounts of RNA, less than 5% of the total. The solids from the ethanol precipitation were digested with 0·33N-NaOH for 18 hr. at 37°. Glacial acetic acid was added to pH 4·0 followed by 95% ethanol (2·5 vol.). After 2 hr. at −20°, the suspension was spun at 2500*g*. The pellet was dissolved in 0·1N-NaOH and heated to 80° for 30 min. The DNA was again precipitated with acetic acid and ethanol, and the two supernatants were combined. The DNA precipitate was washed with 70% (v/v) ethanol and dried.

The supernatants from the alkaline digestion of DNA were evaporated to dryness and the hydrolysed RNA was separated from the sodium acetate by adsorption on Dowex 2 resin in tris–HCl buffer (pH 7·8; 0·1M) and eluted with N-HCl (de Deken-Grenson & de Deken, 1959). This eluate was used for the assay of radioactivity and for the electrophoretic separation of the nucleotides, as described below.

Estimation of deoxyribonucleic acid. DNA was estimated by the method of Burton (1956). A reference preparation of calf-thymus DNA was used as standard. This preparation contained 7·31% of P and after hydrolysis in $HClO_4$ (0·5N at 70° for 30 min.) had $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 232 mμ

and $E^{266}_{1\,cm.}$ for 1 μg. of P/ml. was 0·285. Perchloric acid extracts of whole cells gave the same value for DNA as did ethanol precipitates obtained after separation of RNA from DNA. Both methods were used for the determination of DNA.

Estimation of ribonucleic acid. The orcinol method of Hurlbert, Schmitz, Brumm & Potter (1954) was used. A preparation of yeast RNA was used as standard. This material contained 7·50% of P. After hydrolysis with $HClO_4$ as above the $\lambda_{max.}$ of the standard preparation was 260 mμ, $\lambda_{min.}$ 230 mμ and $E^{260}_{1\,cm.}$ for 1 μg. of P/ml. was 0·335. Except for experiments on the fate of the radioactive precursor (Table 7), all estimations of RNA concentration and radioactivity were performed on the hot $HClO_4$ total nucleic acid extracts. DNA, allowed to react with orcinol in the same manner, gave an extinction at 670 mμ 9·2% of that of an equivalent amount of RNA. Estimates of RNA in total nucleic acid extracts were corrected for the amount of DNA present, measured by Burton's method, by using this factor.

Estimation of nucleotide concentration. The concentration of nucleotides in the cold $HClO_4$ (acid-soluble) extract was estimated from the extinction at $\lambda_{max.}$ (257 mμ), assuming the $E^{257}_{1\,cm.}$ for 1 μg. of nucleotide P/ml. was 0·330. When cold $HClO_4$ extracts from Krebs II cells, previously incubated for 30 min. with [^{14}C]orotic acid, were treated with charcoal, and the nucleotides eluted and subjected to two-dimensional chromatography (Tsuboi & Price, 1959), they were found to contain predominantly the mono- and di-phosphates of adenosine and uridine, and guanosine monophosphate. Uridine-containing nucleotides accounted for 18–20% of the total ultraviolet-absorbing material in the extract. No orotic acid was detected, even after very brief incubations with the precursor.

Measurement of radioactivity

Protein turnover rates were measured by following the rate of incorporation of uniformly labelled L-[^{14}C]valine (specific activity 8·0 mc/m-mole). As a precursor of nucleotide and nucleic acid [6-^{14}C]orotic acid (specific activity 9·7 mc/m-mole) was used. Its radiochemical purity was checked by paper chromatography (Leone & Scale, 1950). Both labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of cells for extraction of radioactive components. Ascites-tumour cells, incubated with [^{14}C]orotic acid (or [^{14}C]valine) for the required period, were diluted with 10 ml. of ice-cold carrier (1 mM-[^{12}C]orotic acid or 4 mM-[^{12}C]valine in PBS) and transferred to 50 ml. centrifuge tubes with a further 10 ml. of carrier. The tubes were centrifuged at 120g for 5 min. at 0°, the supernatant was discarded, and the procedure for the preparation of cells for analytical studies, described above, was applied. When the final washing fluids were examined, they were free of radioactivity and unlabelled precursors.

Radioactive proteins. The cell material which remained after removal of acid-soluble substances, lipids, RNA and DNA as already described was dissolved in NaOH (0·2N) mixed with excess of [^{12}C]amino acid and reprecipitated with trichloroacetic acid. The precipitated protein was washed successively with acetone, acetone–ether and ether and dried. Plates were prepared for counting either by packing the solid on weighed polythene planchets for infinitely thick samples, or by dissolving the protein in 100% formic acid and spreading it upon aluminium planchets for counting at zero thickness. In order to avoid the troublesome curling of dry protein films, the aluminium planchets were coated with silicone adhesive (Cold-Cure Silastomer; Midland Silicones Ltd., London, W. 1) before use.

Radioactive deoxyribonucleic acid. The DNA extracted by the hot NaCl method (see above) was dried, powdered, spread evenly on Perspex disks of 0·3 cm.^{2} area and counted with a standard thin-window Geiger counter. Some samples of DNA were also hydrolysed in $HClO_4$, adsorbed on charcoal as described below, and counted in the same way as RNA. The two methods gave the same result within the limits of the counting error.

Radioactive ribonucleic acid. In most methods for the extraction of total nucleic acids from tissues, the nucleic acids are obtained as a hydrolysate in $HClO_4$ or trichloroacetic acid. Most of the $HClO_4$ may be removed as $KClO_4$ but even the small amount of residual $KClO_4$ that separates when solutions are dried for counting is sufficient to cause large errors in the radioactive assay. Hurlbert & Potter (1952) neutralized trichloroacetic acid extracts with NH_3 and evaporated them to dryness, but this method also gave variable results (cf. Reid & Stevens, 1957). An alternative method was suggested by Kruh & Borsook (1955). They adsorbed nucleotides on activated charcoal and counted the dried samples of charcoal. According to these authors the charcoal had to be saturated with nucleotide and this placed a severe limitation on the usefulness of the method. In the present investigations the charcoal method could be made completely reliable without recourse to nucleotide saturation.

A stock solution of hydrolysed radioactive nucleic acid was prepared in 0·5N-$HClO_4$. This material had a specific activity of 1·060 μc/m-mole of nucleic acid P. A series of samples of Norit A (British Drug Houses Ltd.) were dispensed into centrifuge tubes (2–40 mg.) and portions of the standard radioactive nucleic acid solution were added (211 μg. of nucleic acid P in 1 ml. of 0·5N-$HClO_4$ to each). In a second series of tubes a fixed amount of Norit (40 mg.) was mixed with various quantities of radioactive nucleic acid hydrolysate. The specific activity of the hydrolysates was varied by dilution of the standard radioactive preparation with various amounts of non-radioactive hydrolysate, and the $HClO_4$ volume was varied between 3 and 8 ml. All samples of charcoal were left for 1 hr. at 0° with occasional mixing and then centrifuged at 2200g for 5 min. at 0°. The supernatant was removed and the unadsorbed nucleotide estimated by ultraviolet absorption. The charcoal was washed at 0° with ice-cold water and then with acetone–ether (1:1) followed by two washes with ether. It is essential to avoid ethanol during washing as this elutes some of the adsorbed nucleotide. The dry charcoal samples were plated on 2 cm.^{2} planchets and counted on a thin-window Geiger counter. The results of this experiment (Table 1) demonstrate that the method gave reliable results over a wide range of specific activities and over a wide range of ratios of nucleic acid to charcoal.

The particular advantage of this method was that no limitation was placed on volume of extract that could be taken for radioactivity assay; 80% of the total extract was usually used for this purpose. The procedure adopted throughout the remainder of this investigation was to adsorb the nucleic acid hydrolysate on about 40 mg. of

Table 1. *Determination of specific radioactivity of a nucleic acid hydrolysate by adsorption on charcoal*

The standard preparation of nucleic acid had a specific activity of 1·06 μc/m-mole of nucleic acid P and this was diluted with various quantities of a non-radioactive hydrolysate before mixing with charcoal. Full details are given under Methods.

Charcoal (mg.)	Total nucleic acid used (μg. of P)	Dilution* factor	Amount of standard† radioactive nucleic acid adsorbed (μg. of P/mg. of charcoal)	Specific radioactivity of standard (μc/m-mole of P)
24·9	412	100	15·98	1·110
25·5	206	100	8·00	1·045
25·0	103	100	4·11	1·085
25·1	41·2	100	1·62	1·095
39·8	206	100	5·17	1·050
60·5	206	100	3·39	1·050
26·0	360	57·3	7·77	1·000
40·7	360	57·3	5·03	1·140
60·2	360	57·3	3·42	1·085
25·1	333	30·9	3·99	1·060
40·9	333	30·9	2·50	1·000
25·8	286	80·1	11·73	1·030
40·3	286	80·1	7·59	1·035
25·6	180	57·3	3·99	1·080

* Expressed as the percentage of the standard radioactive preparation in the diluted solution used for assay (column 2).
† Calculated from the difference in extinction of the solution before and after treatment with charcoal.

charcoal, and to measure the extinction of the solution at 264 mμ both before and after treatment for 1 hr. at 0° with charcoal, which was then washed and counted as above. Originally, a sample of nucleic acid of known specific radioactivity was always carried through the same process and its radioactivity assayed concurrently with the unknown samples. However, experience showed that this precaution was unnecessary. The specific activity of the unknown nucleic acid, expressed as μc/m-mole of nucleic acid P, was calculated from the formula:

$$\text{Specific activity} = \frac{\text{Observed counts/min.} \times \text{wt. of charcoal (mg.)}}{\text{Standard counts/min.} \times \text{area of sample (cm.}^2\text{)} \times \mu\text{mole of P adsorbed}}$$

'Standard counts/min.' refers to the counting rate of a 1 cm.^{2} disk of poly[^{14}C]methyl methacrylate of specific activity 1·0 μc/g. (supplied by The Radiochemical Centre); this was normally 900–1000 counts/min. in the counting equipment used for these studies. This method was also used to measure radioactivity in soluble nucleotide extracts.

It is shown below (see Table 7) that the ratio of specific activities of RNA to DNA when [^{14}C]orotic acid is incubated with Krebs II cells under the standard conditions is approx. 19:1. As the ratio of RNA to DNA in these cells was normally about 1·67:1 (see Table 2), the specific activity of RNA in total nucleic acid extracts was estimated on the assumption that 97% of the total radioactivity measured represented incorporation into RNA.

RESULTS

Growth cycle of encephalomyocarditis virus in Krebs II ascites-tumour cells

The growth cycle of EMC virus in Krebs II ascites-tumour cells has already been described by Sanders (1957) and Sanders *et al.* (1958). We have obtained essentially similar results and these are given here only to demonstrate the characteristics of the system. Under the conditions of incubation described in the paper and with an infectivity ratio of 3 plaque-forming units/host cell there is no detectable haemagglutinin or virus until after 3 hr. at 36°. Thereafter there is a steady increase in intracellular haemagglutinin and in the number of intracellular virus particles up to about 8 hr. (Fig. 1). After this time free virus is released into solution and the host cells die.

In some experiments, some degree of synchrony of virus growth was induced by adding the virus at 0° and leaving it at this temperature for 3 hr., when the temperature was rapidly increased to 36° immediately before incubation. The method was suggested to us by Dr Sanders and when it was used for a particular experiment this is stated in the appropriate table. The effect of synchrony was to increase the rate and shorten the time of virus growth.

Effect of virus infection upon total deoxyribonucleic acid, total ribonucleic acid and total protein of Krebs II cells

No previous results have been reported on the effect of EMC virus infection on the level of DNA, RNA and protein in the Krebs cell, but two previous investigations (Ackermann, 1958; Salzman, Lockart & Sebring, 1959) on the effect of polio-

myelitis-virus infection in HeLa cells suggested that quite substantial changes might be found. A series of infected suspensions of Krebs cells were therefore incubated at 36° for periods of from 1 to 7 hr. After the appropriate time, the cell suspension was cooled to 0°, together with a similar uninfected control, and the cells were collected, washed and analysed as described under Methods. One infected and one control flask were incubated for 20 hr. At this time 95% of the infected cells were dead (as shown by staining with nigrosin) while the control flask contained 14% of dead cells. From Table 2, it is evident that there is no change in total RNA, total DNA or total protein as a result of virus infection.

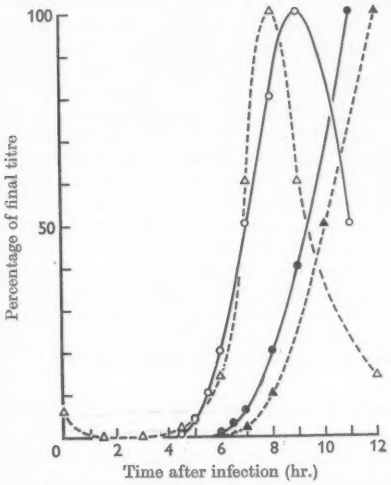

Fig. 1. Growth cycle of EMC virus on Krebs II ascites-tumour cells suspended in Earle's medium. △, Intracellular haemagglutinin; ▲, extracellular haemagglutinin; ○, intracellular infective particles (plaque-forming units); ●, extracellular plaque-forming units.

Effect of virus infection on penetration of [^{14}C]orotic acid into Krebs cells

Radioactive orotic acid was used as tracer in order to compare the rate of turnover of nucleic acid in normal and in infected tumour cells. Since the values for turnover rates measured in this way would be affected by the rate of penetration of radioactive tracer into the intracellular pool of nucleotide, it was essential to ensure that virus infection did not prevent free penetration of the tracer into the cells.

The choice of orotic acid rather than a nucleotide as tracer for nucleic acid turnover was dictated by considerations of cost. It has been suggested, however, that orotic acid is poorly converted into uridylic acid and into RNA in HeLa cells (Pileri & Ledoux, 1957), and it was therefore necessary to examine this point in Krebs II ascites-tumour cells. A series of flasks, each with 1×10^8 cells in Earle's medium, were incubated at 36° for 3 hr. At the end of this time [^{14}C]orotic acid was added and flasks were removed at intervals of 30 min. during the next 2 hr. The intracellular nucleotide pool and the total cell RNA were isolated as described under Methods and their specific radioactivities were determined. From the results given in Table 3, it is evident that orotic acid is a satisfactory label for nucleotides and for RNA in the Krebs cell, and that equilibration between added orotic acid and the intracellular nucleotide pool required less than 30 min.

In another experiment, bulk suspensions of virus-infected and control cells were incubated under the standard conditions for 7 hr. At hourly intervals suitable portions of each cell suspension were transferred to smaller flasks and incubated with [^{14}C]orotic acid for 30 min. At the end of this time ice-cold carrier was added and the cells were washed as already described. Each acid-soluble nucleotide pool was measured quantitatively and its specific radioactivity determined (Table 4). For the first 4 hr., virus infection caused only a slight

Table 2. *Effect of virus infection on ribonucleic acid, deoxyribonucleic acid and protein content of Krebs II cells*

The ascites-tumour cells were suspended in Earle's medium (2×10^7 cells/ml.) and infected with 3 plaque-forming units/cell. Each flask contained 5 ml. of cell suspension. Flasks were incubated at 36° and removed at hourly intervals, and the cells analysed as described in the text.

Time after infection (hr.)	RNA P/10^8 cells (μg.)		DNA P/10^8 cells (μg.)		Protein/10^8 cells (mg.)	
	Infected	Control	Infected	Control	Infected	Control
1	367	375	230	229	30·5	31·9
2	374	371	232	234	30·1	29·4
3	363	373	228	229	29·7	29·8
4	359	356	230	226	30·6	29·6
5	359	368	227	229	30·1	29·1
6	372	376	234	233	30·8	29·1
7	366	386	228	229	30·2	30·9

increase in the size of the nucleotide pool, but during the last 2 hr. there was some loss of nucleotide from the infected cells. The specific activity in the control cells remained almost constant during 6 hr. incubation, but at 4 hr. the specific activity of the pool in infected cells began to rise, and this rise continued steadily until it was more than 100% above the initial value. The very slight lowering in specific activity of nucleotides in the infected cells 1 and 2 hr. after infection is due to dilution of the pool and not to any effect on the rate of precursor incorporation, as the total radioactivity found in the pool is not decreased by infection. It is evident from these results that orotic acid is an adequate precursor of soluble nucleotides in both normal and infected cells and that infection far from causing any inhibition of the rate of conversion of orotic acid into nucleotide actually appeared to stimulate conversion. The significance of these observations became more apparent as further information was collected on the behaviour of infected cells (see below).

Table 3. *Radioactive orotic acid as a precursor of soluble nucleotides and of ribonucleic acid in Krebs II ascites-tumour cells*

The cells (10^8) in Earle's medium (5 ml.) were incubated at 36° for 3 hr. before addition of [6-^{14}C]orotic acid (5 μc). Incubation was continued for the times indicated, and the acid-soluble nucleotides and RNA were then separated and assayed as described in the text.

Time of incubation with orotic acid (min.)	Total radioactivity in nucleotide pool (μmc)	Total radioactivity in RNA (μmc)
30	15·7	15·0
60	13·2	25·1
90	16·4	41·8
120	16·6	58·7

Effect of virus infection on penetration of valine into Krebs cells

To examine the rate of penetration of radioactive valine into the intracellular amino acid pool, a series of suspensions of infected cells were incubated for different times in the presence of [^{14}C]-valine. The cells were then cooled quickly at 0°, washed once with ice-cold 0·9% sodium chloride and homogenized in cold ethanol–water (4:1). The radioactivity of the ethanol-soluble material was taken as a measure of the rate of penetration of valine into the intracellular amino acid pool. From the results in Table 5, it is evident that the added [^{14}C]valine equilibrated with the intracellular valine in about 5 min. In further experiments, virus-infected cells were incubated under the standard conditions and, at various times after infection, [^{14}C]valine was added to an infected flask and to a corresponding control. After a further 5 min. at 36°, the radioactivity of the intracellular pool was measured as above. From the results given in Fig. 2, it is evident that virus infection had little if any effect on uptake of valine into the cell during the first 4 hr. (replicate flasks gave values differing by about 10%). From 6 hr. onwards, however, the cells had lost much of their capacity for the concentration of valine. There seems no doubt that this is a concentrative process since after 5 min. 14% of the ^{14}C added was found in the intracellular pool, whereas the total cell volume only represented about 3% of the total volume (cf. Christensen & Riggs, 1953).

Effect of virus infection on the turnover of ribonucleic acid in Krebs II ascites-tumour cells

In a preliminary experiment, tumour cells were incubated with [^{14}C]orotic acid for 2 hr. under the conditions given in Table 6. At the end of this time

Table 4. *Effect of virus infection on the size and on the radioactivity of the nucleotide pool during incubation with* [^{14}C]*orotic acid*

A large batch of cells (16×10^8 in Earle's medium) was cooled to 4° and divided into two lots, and one lot was infected with virus (3 plaque-forming units/cell). After 3 hr. at 4° the cells were collected, resuspended in fresh Earle's medium (2×10^7 cells/ml.) at 36° (synchronized infection) and incubated in the usual way. At the times indicated samples (5 ml.) were withdrawn from each flask and incubated with [^{14}C]orotic acid (7·5 μc) for 30 min. After this time excess of ice-cold [^{12}C]orotic acid was added and the cells were washed with cold buffer. The intracellular acid-soluble nucleotides were isolated and assayed for specific radioactivity as described in the text.

Time after infection (hr.)	Nucleotide pool size (μmoles of nucleotide P/10^8 cells)		Specific radioactivity of nucleotide pool (μc/m-mole of P)		Infected / Control (specific radioactivity)
	Infected	Control	Infected	Control	
1	1·98	1·88	9·33	9·80	0·95
2	2·19	1·83	8·12	8·71	0·92
3	2·18	1·85	8·59	7·51	1·14
4	2·03	1·82	11·1	8·38	1·32
5	1·99	1·81	14·2	8·08	1·76
6	1·50	1·69	19·5	8·01	2·44
7	1·12	1·76	20·3	6·54	3·10

Table 5. *Rate of equilibration of added* [^{14}C]*valine with the intracellular amino acid pool during incubation of Krebs II ascites-tumour cells in Earle's medium*

Each flask contained 4×10^7 cells in 2 ml. of Earle's medium. Cells were incubated for 1 hr. at 36° before adding valine (0·1 μc; 0·17 μmole). After the stated time cells were rapidly cooled to 0°, washed with cold 0·9% NaCl and homogenized in ethanol–water (4:1, v/v).

Incubation time with [^{14}C]valine (min.)	Radioactivity in ethanol-soluble fraction (counts/min.)	Calculated percentage of added ^{14}C in intracellular valine pool
0	146	0·78
0·5	1240	6·5
1·0	1710	9·0
2·0	2180	11·5
5·0	2680	14·1
10·0	2680	14·1
15·0	2390*	12·6

* The drop in the amount of [^{14}C]valine found in the ethanol-soluble fraction at 15 min. is probably caused by its rapid incorporation into protein.

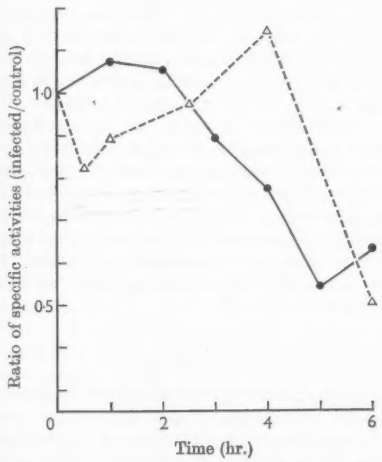

Fig. 2. Effect of virus infection on rate of penetration of valine and on protein turnover in Krebs II ascites-tumour cells. Experimental conditions were as described in the text. Results are expressed as the ratios of specific activities (infected/control) for protein (●), and as ratio of the total radioactivities (infected/control) of free valine in the intracellular pool (△). Intracellular haemagglutinin could not be detected until 4 hr. after infection. At 5 hr. there were 1500 haemagglutinin units and at 6 hr. 56 000. At 7·5 hr. the rate of penetration of valine into infected cells was less than 20% of the control rate and at 8 hr. it was about 7% of the control.

Table 6. *Distribution of radioactivity in the ribonucleic acid and deoxyribonucleic acid of Krebs II tumour cells after incubation with* [^{14}C]*orotic acid*

The Krebs cells (in two flasks, each with 10 ml. of a suspension of 4×10^7 cells/ml. in Earle's medium) were incubated for 2 hr. at 36° with 20 μc of [^{14}C]orotic acid in each flask. RNA and DNA were isolated as described in the text. Both were hydrolysed (see Methods) and the bases separated as described. The only radioactive products were uridine monophosphate from RNA and thymine from DNA.

Material	Radioactivity (μc/mole of P or μc/mole of thymine) Flask 1	Flask 2
RNA	9·9	9·15
DNA	0·547	0·485
UMP	—	33·1
Thymine	—	1·6
RNA/DNA	18·1	18·9
UMP/thymine	—	20·6

RNA and DNA were isolated from the cells by the modified Davidson–Smellie procedure (see Methods) and their specific radioactivities were determined. A sample of the DNA was hydrolysed with formic acid (Wyatt & Cohen, 1953) and the bases were separated in propan-2-ol–hydrochloric acid (Wyatt, 1951). A sample of the hydrolysed RNA was purified as already described and separated into nucleotide components by electrophoresis (Davidson & Smellie, 1952). Each component was assayed for radioactivity with the double-window paper scanner described by Piper & Arnstein (1956). The results (Table 7) showed that the uridine monophosphate from RNA accounted for 97% of the total nucleic acid radioactivity; the remainder was as [^{14}C]thymine in DNA.

In further experiments the rate of RNA turnover in normal cells was compared with that of virus-infected cells. Cells were incubated either with or without virus for various times before addition of [^{14}C]orotic acid. Incubation was continued for a further 30 min. to allow equilibration between the added ^{14}C and the intracellular nucleotide pool. At 30 min. the cell suspensions were cooled to 0° and the acid-soluble nucleotides and nucleic acids were isolated. As shown in Table 7, the rate of incorporation of orotic acid into RNA of control cells fell slowly throughout the incubations. In infected cells, the fall was at first more rapid but at about 5 hr. after infection the rate of incorporation began to increase. By 7 hr. after infection the amount of [^{14}C]orotic acid converted into RNA was more than twice as large in the infected as in the control cells. Much of this increased conversion is due to the increase in specific activity of the soluble nucleotide pool, but even when the results are expressed as the ratio of specific activities of RNA to soluble nucleotides (i.e. relative specific activity),

Table 7. *Effect of virus infection on the rate of turnover of ribonucleic acid in Krebs II ascites-tumour cells*

Specific activity of the RNA from cells used in the experiment described in Table 4 was measured as described in the text. Since there is no net change in the amount of RNA (Table 2) the results are a measure of the rates of turnover.

Time after infection (hr.)	Specific activity of RNA (μc/m-mole of P)		Total uptake of orotic acid (μmc/10^8 cells)		Relative specific activity of RNA*	
	Infected	Control	Infected	Control	Infected	Control
1	2·07	2·57	35·9	39·7	0·222	0·262
2	1·10	1·51	26·9	28·4	0·137	0·173
3	0·575	1·12	23·5	23·2	0·067	0·149
4	0·450	1·04	26·2	23·9	0·041	0·124
5	0·715	0·945	34·0	22·4	0·051	0·116
6	1·13	0·990	40·1	21·7	0·068	0·123
7	1·74	0·795	37·1	18·1	0·086	0·122

* Relative specific activity is calculated and is the ratio between the specific activity of RNA and the specific activity of the nucleotide pool from the same cells.

Table 8. *Effect of infection on the rate of amino acid incorporation into ascites-tumour-cell protein*

Flasks containing normal and infected (3 plaque-forming units/cell) suspensions of tumour cells (2·0 ml.; 2×10^7 cells/ml.) in Earle's medium were incubated at 36° for the periods indicated, and [^{14}C]valine (0·3 μc; 0·17 μmole) was added. Trichloroacetic acid (30%, w/v; 2·0 ml.) was added 30 min. later, the protein isolated, and its radioactivity was determined as described under Methods. Results are expressed as a ratio (infected/control) of specific activities.

Time of incubation with virus (hr.)	Specific activity of protein $\left(\frac{\text{infected}}{\text{control}}\right)$
0·5	0·96
1	1·10
2	1·06
4	0·93
6	0·75
7·5	0·68
8	0·31

a marked stimulation during the 4–7 hr. period in the rate of RNA synthesis is evident. The rate of increase of the relative specific activity during this period was proportional to the increase in intracellular virus titre. However, the amount of new RNA synthesized, calculated from these radioactivity data, seemed to far exceed the total increase in viral RNA calculated from the haemagglutinin titre and the data given by Faulkner *et al.* (1960).

Effect of virus infection on protein turnover in Krebs II ascites-tumour cells

In the experiment described in Table 7, infection was synchronized by the technique developed by Sanders (see under Methods). In another similar experiment incubation was started 30 min. after addition of virus. In the synchronized culture nearly all the infected cells died after 8–8·5 hr. at 36° but in the unsynchronized culture cell death was spread over a 4 hr. period (8–12 hr.). As might be expected, the increase in RNA turnover during the later stages of infection was much less obvious but it was still apparent. In several similar experiments, protein turnover was also measured by incubation with [^{14}C]valine. There was a slow fall in the rate of protein turnover in control cells throughout the 8 hr. incubation period. The rate of protein turnover fell more quickly in the infected cells and at 8 hr. was about 30% of that of the controls (Table 8). We have already shown, however, that the capacity of the cell to concentrate valine is lost during the later stages of infection (Fig. 2) and it may well be that any synthesis of viral protein would be obscured by this factor. Even if no allowance is made for this factor, however, a plot of rate of protein turnover in infected cells against that in control cells showed that there was some stimulation of protein synthesis in the infected cells around the sixth hour after infection (Fig. 2).

DISCUSSION

Much attention has been devoted by Cohen and others to delineation of the biochemical events which accompany reproduction of bacteriophage within *Escherichia coli* (Cohen, 1957; Flaks, Lichtenstein & Cohen, 1959). Less attention has been paid to the related biochemical problems which arise during virus replication within mammalian cells. The slower rate of progress in the mammalian field has been occasioned by both quantitative and qualitative factors. With a virus such as EMC, the amount of virus material that is formed in the host cell in a single growth cycle is an extremely small percentage of the total mass of the host cell. For this reason quantitative changes in host-cell composition are difficult to detect, and in

fact there were no measurable changes in overall content of RNA, DNA and protein in Krebs II cells during a complete cycle of virus growth.

In addition, mammalian RNA viruses do not contain, so far as is known, any base residues such as 5-hydroxymethylcytosine, and the formation of viral RNA cannot therefore be followed by qualitative methods.

No comparable results are available from other Laboratories on Krebs cells but the related system of poliomyelitis-virus growth in HeLa-cell suspension has been studied in some detail. The results from different Laboratories are contradictory, probably because the HeLa cell deteriorates in a simple maintenance medium such as that used for Krebs cells and it is thus difficult to distinguish between damage caused by virus infection and natural deterioration of the host cell. Ackermann, Loh & Payne (1959) found that in HeLa cells infected by polio virus there was a large increase of RNA in the cytoplasm during the first 4 hr. after infection, but Salzman *et al.* (1959) found no change in total RNA, DNA or protein during the first 3 hr. and thereafter a loss of RNA from the infected cells. These effects are reproduced by incubation of cells in an amino acid-deficient medium and the loss of RNA is at least three times as great as would be required for virus synthesis. These results indicate the difficulty of working with a cell that has to be maintained in a complex medium and underline the advantages offered by the Krebs ascites-tumour cell.

Since no quantitative changes in RNA, DNA or protein were detected during the growth cycle of EMC virus in Krebs cells it became necessary to use radioactive tracers in order to detect changes in nucleic acid and protein metabolism. Although ^{32}P has been used in several investigations on the effect of poliomyelitis-virus infection (Miroff, Cornatzer & Fischer, 1957; Ackermann *et al.* 1959; Contreras, Tohá & Ohlbaum, 1959), we regarded it as unsuitable for such investigations since it is incorporated into a wide variety of cell components. It is thus difficult to know the nature of the radioactivity of the particular phosphate pool that will be used for synthesis of any particular type of molecule, and, moreover, it is notoriously difficult to remove adsorbed radioactive phosphates of various kinds from RNA and DNA (Davidson & Smellie, 1952). It is clear from our results, however, that [^{14}C]orotic acid is a suitable tracer, since virus infection does not inhibit uptake and the orotic acid is rapidly converted by the Krebs cell into uridine nucleotides. It is thus possible to measure the radioactivity of the nucleotide pool and to distinguish between effects of virus infection on nucleotide metabolism and on RNA metabolism. To illustrate the effect of virus infection upon nucleotide and upon RNA metabolism, the results of the experiments outlined in Tables 4 and 7 are presented graphically as Fig. 3. It may be suggested that the cycle of events after virus infection is roughly as follows. During the first 4 hr. after infection there is an overall inhibition of RNA synthesis with a consequent accumulation of small nucleotides. From 4 hr. onwards and almost coincident with the appearance of intracellular virus there is an increase in the rate of synthesis of new RNA. Since there is probably no overall increase in RNA this synthesis is likely to be accompanied by an accelerated loss of old RNA, probably cell RNA. The fall in the size of the nucleotide pool indicates that the new RNA is being synthesized direct from nucleotides, and the rapid rise in the specific activity of this pool when radioactive orotic acid is added to the incubation medium indicates that the permeability barriers within the cell have begun to break down so that orotic acid can enter the nucleotide pool more quickly than in the normal cell. Before a more detailed picture of these biochemical events can be presented it will be necessary to study the changes that occur within the various intracellular organelles and to distinguish

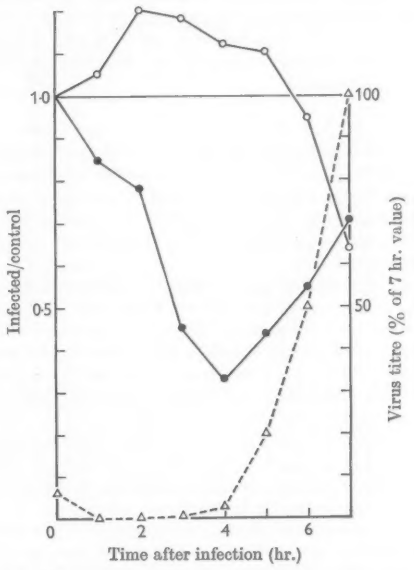

Fig. 3. Effect of virus infection on the rate of turnover of RNA in Krebs II ascites-tumour cells. Relative specific activity is defined as the ratio of specific activity of RNA to that of the nucleotide pool measured at the same time. △, Intracellular virus haemagglutinin titre; ●, RNA; ○, size of intracellular-nucleotide pool.

between synthesis of virus RNA and synthesis of other types of RNA.

The results obtained by the measurement of protein turnover in intact cells have been less conclusive than those for RNA. The mechanism for amino acid concentration by Krebs cells begins to break down some time between 4 and 6 hr. after infection and this breakdown probably serves to obscure the increase in protein turnover which would be expected to accompany virus synthesis. There is, nevertheless, some increase in the rate of protein turnover about 5 hr. after infection, which may represent synthesis of viral protein. With further development of our technique, virus has been isolated at various stages during the growth cycle and the results obtained in this way (E. M. Martin & T. S. Work, unpublished work) are in general agreement with our present interpretation.

In all the numerous experiments that were performed, a small increase in the rate of protein turnover in infected cells was observed between 1 and 2 hr. after infection (cf. Fig. 2). This increase was never more than about 20 % above that in the control, and we were at first inclined to regard it as insignificant. With constant repetition, however, we now believe that there is a genuine stimulation of protein turnover in infected cells during this period, and it is tempting to suppose that this may be related to the requirement for preliminary synthesis of new enzymes before virus reproduction can commence.

SUMMARY

1. Conditions are described for the propagation of Krebs II ascites-tumour cells and for measurement of the effects of infection by encephalomyocarditis virus on these cells in circumstances favourable for quantitative biochemical investigation.

2. The rate of penetration of orotic acid and of valine into Krebs cells was measured. Infection caused no alteration in the rate of penetration of orotic acid during the first 4 hr. During the later stages of the infectious cycle. orotic acid was taken up at a progressively increased rate, and cells lost their capacity to concentrate valine.

3. During the period of rapid virus synthesis there was a marked increase in the rate of ribonucleic acid turnover and a decrease in the size of the intracellular nucleotide pool.

4. Virus synthesis was accompanied by a small increase in the rate of protein turnover, but this effect was obscured by the loss of capacity to concentrate the radioactive tracer (valine) used to measure turnover rate.

5. In all experiments the infected cells showed a small increase in the rate of protein turnover during the first 2 hr. after infection and before any synthesis of new virus could be detected. This stimulation may be associated with the need for synthesis of new enzymes before virus reproduction can commence.

6. There was no change in the total deoxyribonucleic acid, ribonucleic acid or protein in infected cells.

7. A method of haemagglutinin assay was developed which gave results within 30 min. A method for the measurement of the radioactivity of nucleotides present in aqueous acid extracts of tissues is also described.

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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

2. THE ISOLATION, CRYSTALLIZATION AND CHEMICAL CHARACTERIZATION OF MOUSE ENCEPHALOMYOCARDITIS VIRUS

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Encephalomyocarditis virus is one of the large group of animal viruses, the Col SK group, rather closely related to poliomyelitis virus. They are generally pathogenic to rodents and occasional infection occurs in man (Jungeblut, 1958). The strain that has now been characterized was obtained through the courtesy of Dr Kingsley Sanders (cf. Hoskins & Sanders, 1957). It is highly pathogenic to mice but, after growth for many generations in Krebs II ascites-tumour cells (Martin, Malec, Sved & Work, 1961*b*), it was incapable of causing infection in rabbits even when massive doses of concentrated virus were injected intracerebrally.

Since we had studied the effect of infection on the nucleic acid and protein turnover of mouse-ascites-tumour cells, and had found that virus infection caused striking changes in the pattern of nucleic acid turnover (Martin *et al.* 1961*b*), we wished to extend these studies and to determine, if possible, the site of synthesis of virus nucleic acid and virus protein within the host cell. As part of this project it became necessary to characterize the virus more fully, and in the present paper we describe methods for large-scale culture of the virus and for purification and crystallization of the infective particle. The virus is, in the dry state, a spheroid of about 27 mμ diameter. Its nucleic acid and protein have been analysed. A convenient method has also been developed for the preparation of a precipitating antibody of high titre which may be used to flocculate or neutralize the virus. A preliminary report of some of this work has been published (Faulkner, Martin, Sved & Work, 1960).

METHODS

Abbreviations. EMC, Encephalomyocarditis; DEAE, diethylaminoethylcellulose.

Protein estimation. The method of Lowry, Rosebrough, Farr & Randall (1951) was scaled down to a total volume of 0·9 ml. and the extinction of the product was measured at 750 mμ ($E_{1\text{ cm.}}$) against a reagent blank. The method was satisfactory over the range 1–20 μg. of protein. Extinction measurements were made on 0·8 ml. of solution in normal 1 cm. cuvettes fitted with Perspex displacers, as described by Martin & Morton (1956). Since no standard preparation of virus protein was available the method was standardized on host-cell protein prepared as already described (Martin *et al.* 1961*b*).

Total nitrogen. The method of Jacobs (1960) was used. In this method, N is converted into NH_3 and estimated by interaction with nmhydrin. It is sensitive to 1 μg.

Total phosphorus. The method of Griswold, Humoller & McIntyre (1951) was used. It was satisfactory with 0·5–5 μg. of P in a final volume of 3·5 ml.

Deoxyribonucleic acid. The method of Burton (1956) was scaled down to a final volume of 0·9 ml. Satisfactory results were obtained with 1–10 μg. of DNA.

Ribonucleic acid. The orcinol method of Hurlbert, Schmitz, Brumm & Potter (1954) was used. The nucleic acid sample (0·3 ml.) was mixed with 0·6 ml. of orcinol reagent (0·4% of orcinol and 0·033% of $FeCl_3$, w/v, in 12 N-HCl) and heated at 100° for 30 min. Extinction was measured at 675 mμ in microcells (1 cm. path); the satisfactory range was 2–20 μg. of RNA.

Total nucleic acid. Samples were hydrolysed in 0·5 N-$HClO_4$ at 70° for 30 min. Extinction was measured (after centrifuging) at 260 mμ and ϵ_P was taken as 10 800 (Beaven, Holiday & Johnson, 1955).

Base analysis of ribonucleic acid. Samples were hydrolysed in N-HCl as described by Smith & Markham (1950), and the products, guanine, adenine, cytidylic acid and uridylic acid, separated by chromatography in *tert.*-butyl alcohol–HCl (cf. Markham, 1954). Individual bases were eluted in 0·1 N-HCl and the ultraviolet absorptions of the eluates measured at the appropriate wavelengths. The method gave reproducible results with 250 μg. samples of RNA.

Haemagglutinin assay. The method has been described by Martin *et al.* (1961*b*). In addition to the plate technique, the rapid haemagglutinin assay was particularly useful in following individual steps during the purification of the virus.

Plaque counts. The method of Sanders (1957) was used as already described (Martin *et al.* 1961*b*).

Particle counts. The number of virus particles/ml. was determined by the method of Backus & Williams (1950). The virus suspension was mixed with an equal volume of a suspension of polystyrene latex in the form of spherical particles of a very uniform diameter (0·188 μ); such suspensions were generously supplied by the Dow Chemical Co., Midland, Mich., U.S.A. (Bradford & Vanderhoff, 1955). The latex-particle count was known from the particle size and density and the dry weight of the suspension. The electron microscope was then used to determine the ratio of latex to virus-particle count in the mixed suspension, and the count of virus particles/ml. thus obtained. Sufficient particles were counted to give an accuracy of about 25%. (Breese & Trautman, 1960).

Electron microscopy. The virus preparations were examined by the negative-staining method of Brenner & Horne (1959) by a slightly modified technique (Valentine, 1959).

Chromatography on calcium phosphate. Calcium phosphate was prepared by the method of Taverne, Marshall & Fulton (1958). When examined microscopically, freshly prepared calcium phosphate consisted predominantly of spherical microcrystalline aggregates with lesser amounts of relatively larger rhombic crystals. Preparations containing a higher proportion of this latter crystalline material had poorer packing properties and columns prepared from them tended to contract, thus slowing down the eluent-flow rate. The proportion and size of these crystals increased with storage. Therefore, the calcium phosphate was stored at 0° and used within 3 weeks of preparation.

The calcium phosphate was suspended in 0·02 M-sodium phosphate buffer (pH 7) and poured into a column (diameter 25 mm.) fitted with a glass-sintered bottom. Sufficient was added to give a final packed column of 8 cm. The solid was packed under pressure (5 cm. Hg) and washed with 0·02 M-buffer (100 ml.). Trypsin-treated virus suspension (10 ml.), and containing 100–200 mg. of protein, was diluted with an equal volume of 0·02 M-buffer and added to the column. The flow rate was maintained at about 2 ml./min. and the virus preparation was washed in with 10–20 ml. of 0·02 M-buffer. The elution gradient was developed by siphoning 0·5 M-sodium phosphate buffer (pH 7) from a 175 ml. conical flask into an open cylindrical mixing chamber of 5 cm. diameter (cf. Boman, 1955). Mixing was started with 90 ml. of 0·02 M-buffer in the chamber and the liquid level equal in the two vessels. The first 30 ml. of effluent was collected in one lot and thereafter 30 fractions of 5 ml. were collected. Rapid haemagglutinin assays were performed in each fraction (diluted 1:1000) and those fractions that were obviously rich in virus were assayed more accurately by the standard haemagglutinin method (Martin *et al.* 1961*b*). Fractions were stored at 0° overnight.

Those fractions with a haemagglutinin titre above 25 000 were pooled and dialysed against 0·02 M-phosphate on a rocking dialyser for 3 hr., and the dialysed material (usually about 35 ml.) was applied to a second calcium phosphate column and washed in with 50 ml. of 0·02 M-buffer. The second column (diameter 15 mm.; length 10 cm.) had a flow rate of 0·5 ml./min. A steep elution gradient of phosphate was established by adding 60 ml. of 0·02 M-phosphate (pH 7) to the cylindrical mixing chamber and by siphoning into this 90 ml. of 0·5 M-sodium phosphate (pH 7) from a 100 ml. standard volumetric flask. The first 35 ml. of effluent was collected as one batch and the remainder in 2·5 ml. fractions. The rapid haemagglutinin assay was again used to locate those fractions rich in virus.

RESULTS

Large-scale growth of virus

The conditions adopted were essentially those given by Martin *et al.* (1961*b*). For good virus growth in the Krebs II mouse-ascites-tumour cells, it was best to suspend the cells in Earle's medium at a concentration of 2×10^7 cells/ml. at room temperature and to add 3 plaque-forming units of virus/cell. After 30 min. the flasks were transferred to a rotary shaker (70 cyc./min.) and incubated for 18 hr. at 36°. After this time the number of live cells was below 5% and (after removal of the cells) the haemagglutinin titre of the supernatant was about 25 000. It was necessary to maintain a fixed ratio of liquid volume to gas space in the culture flasks and 'penicillin culture flasks' (Laboratory Glassblowers Co. Ltd., High Wycombe, Bucks.) gave excellent results. These flasks are cylindrical, of 17 cm. in diameter and 9 cm. in height, and each flask held 100–150 ml. of Earle's medium.

Partial purification with Arcton 63

Several viruses have been purified by extraction with a fluorocarbon (Gessler, Bender & Parkinson,

1956; Manson, Rothstein & Rake, 1957; Hummeler & Hamparian, 1957). The method depends upon the resistance of virus nucleoprotein to surface denaturation. A suspension of EMC virus prepared as described above was separated from dead cells by centrifuging for 10 min. at 8000*g* and the supernatant from this recentrifuged at 100 000*g* (Spinco) for 1 hr. The Spinco pellet was resuspended in phosphate-buffered saline (Martin *et al.* 1961*b*) and this crude virus preparation was homogenized with the fluorohydrocarbon (Arcton 63; Imperial Chemical Industries Ltd.) as described in Table 1. At each extraction, a large amount of denatured protein was discarded but there was also a considerable loss of haemagglutinin. Moreover, the method did not distinguish between virus particles and ribosomes (the RNA-rich particles of the microsomal fraction of ascites-tumour cells). A batch of uninfected cells, disrupted by ultrasonics, gave a microsome fraction, which was extracted with Arcton in the same way as the virus culture. The product had virtually the same analytical composition as the Arcton-purified virus (Table 2). Thus Arcton treatment will readily purify the virus about 25-fold, but it will also carry along with the virus non-viral ribonucleoproteins from the host cell.

Table 1. *Purification of encephalomyocarditis virus by extraction with Arcton* 63

The virus culture (80 ml.; 1×10^9 cells) was prepared as described in the text. Cells were removed at 8000*g* (10 min.) and the virus was spun down at 100 000*g* (1 hr.). The pellet was homogenized with 10 ml. of phosphate-buffered saline (Martin *et al.* 1961*b*) in a Potter homogenizer and then with an equal volume of Arcton 63 in a sealed blender at 20 000 rev./min. for 15 sec. Denatured protein separated at the interface and was removed; the virus was spun down and resuspended, and Arcton treatment was then repeated. The virus concentration in each fraction is expressed as haemagglutinin units/ml.

Fraction	Concn. of virus
Culture fluid (80 ml.)	6 400
100 000*g* supernatant (80 ml.)	500
Resuspended pellet (10 ml.)	25 000
Arcton treated (10 ml.)	50 000*
Resuspended pellet (2 ml.)	128 000
Arcton treated (2 ml.)	100 000

* The apparent increase in haemagglutinin after the first Arcton treatment is probably due to removal of phospholipid (Porterfield & Rowe, 1960).

Partial purification of virus by combined proteolysis and centrifuging

Weil, Warren, Breese, Russ & Jeffries (1952) reported that EMC virus, partially purified from mouse brain, was resistant to digestion with either trypsin or chymotrypsin. Since proteolytic digestion would be expected to remove at least some of the contaminating protein the effect of these two enzymes on EMC virus from ascites-tumour cells was examined. The supernatant from an 18 hr. infected culture, such as that described in Table 1, was spun first at 8000*g* for 10 min. and then at 100 000*g* for 1 hr. The pellet collected after high-speed centrifuging was resuspended in sodium phosphate buffer (pH 7·1; 0·02 M; 10 ml.), divided into two lots and incubated with either chymotrypsin or trypsin (0·01 % of crystalline bovine pancreatic enzyme) for 1 hr. at 37°. The chymotrypsin-treated preparation lost 97 % of its haemagglutinating activity whereas there was no measurable loss during incubation with trypsin. Treatment with trypsin was therefore adopted as the first step in large-scale purification of the virus.

The yield of virus and the nitrogen content of the trypsin-treated concentrate varied considerably in successive cultures. The mean specific virus concentration of these concentrates (nine preparations) was $2 \cdot 0 \times 10^4$ haemagglutinin units/mg. of protein. This represented an eightfold purification of the virus from the original infected 8000*g* supernatant. The yields varied between 7×10^3 and $2 \cdot 4 \times 10^4$

Table 2. *Analytical composition of virus concentrate and of ribosomes after homogenizing with Arcton*

The crude virus preparation was a culture supernatant from 30×10^8 infected ascites-tumour cells incubated as described in text. The virus concentrate was prepared as in Table 1. The crude microsome fraction was from 34×10^8 tumour cells disrupted by sonic disintegration and was prepared by the method of Martin, Malec, Coote & Work (1961*a*).

	Protein (mg.)	RNA (mg.)	Protein/RNA
Crude virus	300	—	—
Crude microsomes	185	—	—
Concentrated sample of virus* (×4 Arcton extracted)	7·7	5·6	1·37
Ribosome concentrate (×4 Arcton extracted)	6·9	4·4	1·57

* The crude virus contained $0 \cdot 8 \times 10^4$ haemagglutinin units/mg. of protein and the virus concentrate $2 \cdot 0 \times 10^5$ haemagglutinin units/mg. of protein (haemagglutinin units = haemagglutinin titre × vol. in ml.).

haemagglutinin units/ml. of culture fluid and the specific virus concentration varied between $1{\cdot}2 \times 10^4$ and $2{\cdot}8 \times 10^4$ haemagglutinin units/mg. of protein.

Use of modified cellulose ion-exchange columns

The capacity of the two resins DEAE-SF and ECTEOLA-SF (Peterson & Sober,1956) to adsorb virus was measured by adding successive samples of trypsin-treated virus to small columns of the ion-exchange material previously equilibrated with phosphate buffer. Levintow & Darnell (1960) have reported that poliomyelitis virus is not held in ECTEOLA columns and that the resin may be used to remove contaminating protein from the virus. ECTEOLA also appears to have a low capacity for EMC virus but the hold-up of virus was sufficient to render the method unsatisfactory for purification. Attention was devoted instead to the possibility of stepwise or gradient elution of virus from DEAE columns. Columns were equilibrated with phosphate buffer, loaded with trypsin-treated virus and eluted with phosphate containing increasing concentrations of sodium chloride.

The best results were obtained when the virus was eluted by a steeply rising concentration gradient of sodium chloride (Fig. 1), but even under these conditions the virus was eluted over rather a broad concentration range of sodium chloride.

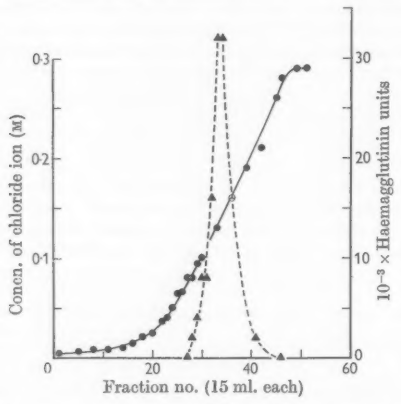


Fig. 1. Partial purification of EMC virus on DEAE. The column (0·8 cm. diameter) contained 1 g. of DEAE in sodium phosphate buffer (pH 7·0, 0·02 M). The column load was 30–60 mg. of trypsin-treated virus. Elution was achieved with a rising concentration gradient of NaCl in sodium phosphate buffer (pH 7·0; 0·02 M). The gradient was established by a method similar to that described for the elution of calcium phosphate columns (see text). The concentration of chloride in the effluents was measured by the method of Schales & Schales (1941). ▲, Haemagglutinin; ●, chloride.

The best preparations contained about 8×10^5 haemagglutinin units/mg. of protein; thus the degree of purification over the crude trypsin-treated preparations was about 40-fold but the virus could not be crystallized.

Purification of virus on calcium phosphate

Calcium phosphate columns showed two important advantages over DEAE: firstly the recovery of virus was usually quantitative and secondly the bulk of the non-viral material failed to adsorb on the column and was immediately separated from the virus. For complete purification of the virus, it was necessary to use two successive columns but as each column run, and the associated virus assays, could be completed in a few hours this was no serious disadvantage. The behaviour of the crude trypsin-treated virus on the first column is given in Fig. 2 and the behaviour of the partially purified virus on a second, smaller, column is shown in Fig. 3. Virus was usually eluted at phosphate concentrations between 0·2 and 0·3 M. These columns also showed some capacity to fractionate nucleic acids present in the crude virus preparation (Vizoso & Burness, 1960).

The mean specific virus concentration (three preparations) in the virus-containing fractions eluted from the first column was 8×10^5 haem-agglutinin units/mg. of protein (i.e. a 40-fold purification from the trypsin-treated concentrate), and, from the second column, was $1{\cdot}5 \times 10^6$ haem-agglutinin units/mg. of protein (i.e. a further three-fold purification).

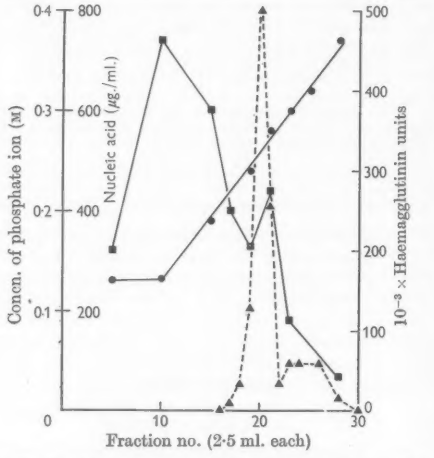


Fig. 2. Partial purification of EMC virus on calcium phosphate (column 1 of Methods). ●, Phosphate; ▲, haem-agglutinin; ■, nucleic acid.

Crystallization of encephalomyocarditis virus

By the use of a steep phosphate gradient as described under Methods, it was possible to elute virus from the second calcium phosphate column in a concentrated and nearly pure form. Those fractions which had a haemagglutinin titre above 50 000 were pooled and centrifuged at 100 000*g* for 60 min. The gelatinous pellets from 10 ml. of the original trypsin-treated virus were combined and resuspended in about 0·2 ml. of 0·9% sodium chloride together with 0·02 ml. of penicillin (50 000 i.u./ml.). After 2 days at 0° crystals had been deposited. The average yield of crystals was about 1 mg./100 ml. of the original Earle's culture medium. The crystals were washed repeatedly with cold water (2 ml./mg. of crystals) and dissolved either in phosphate buffer (pH 8·5; 1 ml.) or in water (1 ml.) to which about 2 drops of 0·06M-sodium carbonate were added. On readjusting to pH 7 and leaving at 0° crystalline virus separated again.

Characterization of crystalline encephalomyocarditis virus

The virus crystallized in the form of extremely thin rectangular wafers. Photographs were taken at wavelengths of 257, 275 and 300 mμ. These photographs (Plate 2) showed clearly that the crystalline material absorbed strongly in the nucleoprotein region and this was confirmed by measuring the absorption spectrum of crystals dissolved in sodium carbonate as described above (Fig. 4). X-ray photographs of crystals have been taken both by Mrs Kennard of this Institute and by Dr Klug and Dr Finch of Birkbeck College. The crystals are extremely fragile and entirely satisfactory photographs have not been obtained, but two conclusions can be drawn from the available photographs: first, the individual particles of virus are almost spherical; and secondly, the particle diameter in the wet preparation is 30 ± 1 mμ.

Electron-microscope photographs of spray preparations of the crystalline material were taken after negative staining with phosphotungstate (see

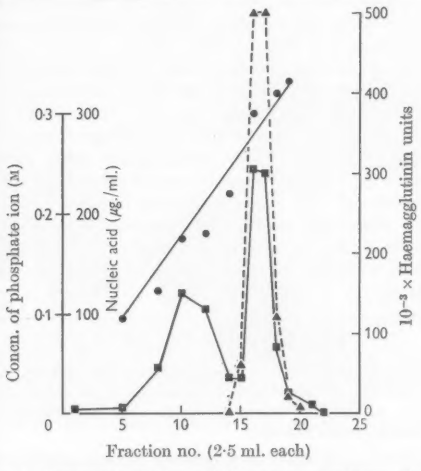


Fig. 3. Preparation of pure EMC virus on calcium phosphate (column 2 of Methods). Symbols as in Fig. 2.

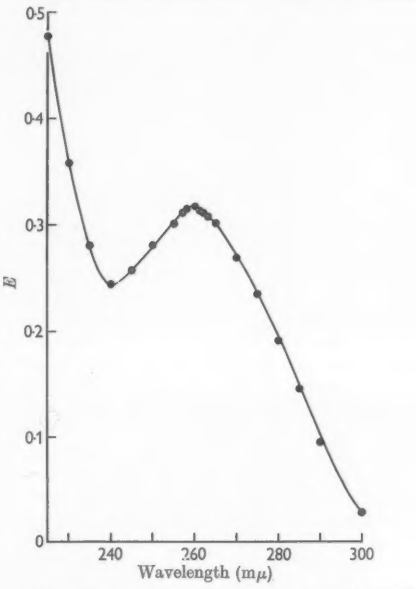


Fig. 4. Absorption spectrum of a solution of purified EMC virus in dilute Na_2CO_3, pH 8·5.

Table 3. *Analysis of crystalline encephalomyocarditis virus for nitrogen, phosphorus and ribonucleic acid*

For methods of analysis see text. Analyses are expressed as μg. of the appropriate component in each standard solution of crystalline virus.

Prep.	N	P	RNA*	RNA† (%)
43	42·5	—	90	33
46	9·2	—	20·7	35
48	220	—	398	28·5
49	273	—	522	30·1
57	160	30	—	32·4

* Spectrophotometric estimation.

† The percentage of RNA was calculated by making the following assumptions: RNA P = 8·75%, RNA N = 15%, protein N = 6·25%. It was also assumed that the virus contained no DNA and no phospholipid since these components were not detected; hence, the estimates represent percentages of total dry weight of the virus.

Table 4. *Haemagglutinin, particle count and infectivity for various preparations of encephalomyocarditis virus*

Where estimations were not made this is indicated by the symbol —. HA, Haemagglutinin units.

Prep.	HA/ ml.	HA/ mg. of N	HA/ mg. of RNA	Particles/ml. (electron microscope)	Infectivity/ml. Plaque	Infectivity/ml. Cell death	Remarks
F30–2	$2{\cdot}4 \times 10^5$	$8{\cdot}8 \times 10^5$	—	6×10^{11}	$3{\cdot}3 \times 10^{10}$	—	Purified in DEAE
F39–2	$5{\cdot}0 \times 10^5$	$4{\cdot}13 \times 10^6$	$2{\cdot}02 \times 10^6$	—	1×10^{11}	—	Calcium phosphate effluent
F40–2	$5{\cdot}0 \times 10^6$	$4{\cdot}74 \times 10^6$	$2{\cdot}32 \times 10^6$	1×10^{15}	$1{\cdot}2 \times 10^{14}$	—	Crystals
F64	$0{\cdot}6 \times 10^4$	$2{\cdot}05 \times 10^6$	1×10^6	—	$1{\cdot}05 \times 10^9$	$1{\cdot}5 \times 10^9$	Crystals stored at $-70°$ for 1 week in Earle's medium
F66	$2{\cdot}5 \times 10^4$	$5{\cdot}0 \times 10^6$	$2{\cdot}45 \times 10^6$	4×10^{11}	$3{\cdot}1 \times 10^9$	$3{\cdot}5 \times 10^9$	Calcium phosphate effluent, centrifuged twice at 100 000*g* for 1 hr.
F66A	$1{\cdot}2 \times 10^4$	—	—	—	$3{\cdot}3 \times 10^9$	—	F66 stored at $-70°$ for 1 week in 10% glucose–phosphate

Methods). Some of these were taken in this Institute and some by Mr Hills (A.R.C. Virus Research Institute, Cambridge). The conclusion from the two sets of micrographs was that the virus particle (Plate 2) resembles turnip yellow virus both in size and in appearance and that, moreover, the virus particle shows a sub-unit structure closely similar to that reported by Nixon & Gibbs (1960). A particle count was also made (see Methods) and this was compared with infectivity measurement by the plaque technique. Three determinations on three separate preparations of crystalline material were made; the best results indicated that about 1 particle in 10 produced a focus of infection. This compares favourably with the ratios (1:30) reported for partially purified poliomyelitis virus by Schwerdt & Schaffer (1956) and is much higher than the ratio (1:2000) found for crystalline poliomyelitis virus.

Analysis of crystalline encephalomyocarditis virus

Four separate preparations of crystalline virus have been analysed by the methods already described, and the results are summarized in Table 3. No virus preparation contained measurable quantities of DNA and all contained around 30% of RNA. Since the RNA accounts for all the phosphorus of the virus (Table 3) the virus must be substantially free of phospholipid.

The relationship between haemagglutinin, infectivity, total RNA and particle count is set out in Table 4.

From the plaque assays made on solutions of crystals that were also analysed for RNA it was calculated that there were about 5×10^{13} infective particles/mg. of viral RNA. Since the virus contains about 30% RNA it may be calculated that 1 g. of virus should contain about $1{\cdot}6 \times 10^{16}$ infective particles or, if allowance is made for the ratio between plaque counts and particle counts, a total of about $1{\cdot}6 \times 10^{17}$ particles. This agrees reasonably well with the figures arrived at by measurement of particle weight in the ultracentrifuge (6×10^{16}, see below) and by measurement of the diameter of the spherical particle with the electron microscope (7×10^{16}).

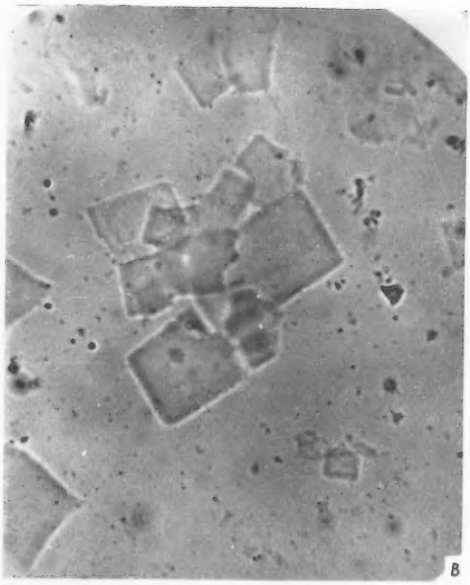
The base ratios for the RNA of crystalline EMC virus were determined by quantitative paper chromatography. Three successive batches of crystals were analysed. The analyses are quite consistent and differ considerably from similar analyses made on host-cell RNA (Table 5). Poliomyelitis-virus RNA is, however, closely similar in base composition to that of EMC virus.

A sample of crystalline virus was also hydrolysed with 6N-HCl at 108° and the amino acid composition of the hydrolysates was determined by our colleague, Dr S. Jacobs, by ion-exchange chromatographic analysis (Moore, Spackman & Stein, 1958). Since the method requires rather large quantities of material only a single analysis has been carried out. Our analysis for EMC virus is compared in Table 6 with a similar analysis of poliomyelitis virus recently published by Levintow & Darnell (1960).

EXPLANATION OF PLATE 2

A. Electron-microscope photograph of a spray preparation of EMC virus immediately after elution from second calcium phosphate column (negative-staining technique; magnification × 140 000).

B, *C* and *D*. Ultraviolet-microscope photographs of EMC virus crystals taken at 300 mμ (*B*), 275 mμ (*C*) and 257 mμ (*D*). Magnification × 2100.



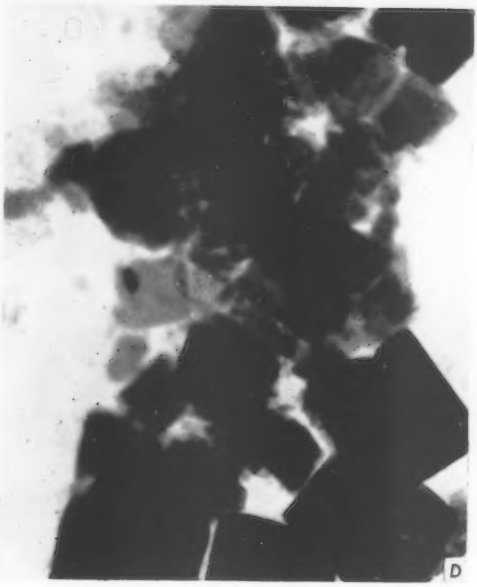


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Table 5. *Base composition of crystalline encephalomyocarditis-virus ribonucleic acid compared with that of various other types of ribonucleic acid*

Figures for EMC virus are for three successive preparations of crystalline material. Figures for poliomyelitis virus are taken from Schaffer, Moore & Schwerdt (1960). Results are expressed as moles of purine or pyrimidine in 100 moles of base recovered after hydrolysis.

				Source of material		
	EMC virus			Krebs II tumour-cell nuclei	Total RNA of Krebs cells	Polio virus
Base	54	55	56			
Guanine	23·7	23·6	23·4	22·4	32·3	24·0
Adenine	27·8	27·3	27·0	24·9	21·4	28·5
Cytosine	23·9	23·2	23·3	28·4	29·1	22·0
Uracil	24·5	26·0	26·4	23·5	17·2	25·4
$\frac{A+G}{C+U}$	1·06	1·03	1·01	0·91	1·16	1·11

Table 6. *Amino acid composition of crystalline encephalomyocarditis virus compared with that of poliomyelitis virus*

The figures for poliomyelitis virus are taken from Levintow & Darnell (1960). Results are expressed as g. of amino acid/100 g. of amino acid recovered.

	EMC	Poliomyelitis
Aspartic acid	11·4	12·2
Threonine	8·1	8·5
Serine	6·6	5·3
Glutamic acid	10·2	8·7
Proline	6·8	7·0
Glycine	5·2	3·9
Alanine	5·0	5·3
Cystine ($\frac{1}{2}$)	1·4	0·7
Valine	6·3	6·6
Methionine	2·3	2·4
Isoleucine	4·1	4·8
Leucine	8·7	8·3
Tyrosine	5·4	6·0
Phenylalanine	6·7	5·7
Lysine	5·2	5·0
Histidine	1·7	2·9
Arginine	4·9	6·6

Sedimentation of purified virus in the analytical ultracentrifuge

Two preparations of crystalline virus were kindly analysed for us by Dr Charlwood of this Institute. Both preparations showed a single component and the two sedimentation constants ($S_{20,w}$) were 160 and 156. For $S_{20,w}$ 160 and a density 1·37 the calculated particle (molecular) weight is 1×10^7.

Stability of crystalline encephalomyocarditis virus

The crystalline virus retains its infectivity for considerable periods if stored as the moist pellet at 4°. For experimental use, however, it was necessary to store the virus as a suspension of known infectivity and methods for the preservation of viral suspensions were therefore investigated. A preparation of crystalline virus was dissolved in 0·02 M-phosphate buffer by careful addition of sodium carbonate solution, and the clear suspension of particles diluted with various media (5 and 50% sucrose, 5 and 50% glucose, 5 and 50% glycerol, 0·1 and 1·0% bovine serum albumin, and Earle's medium) until the haemagglutinin titre of the diluted material was approx. 30 000 haemagglutinin units/ml. Haemagglutinin titre and plaque assay were carried out before and after storage at −70° for 6 months in sealed ampoules. A portion of the suspension diluted in Earle's medium was also freeze-dried and kept at room temperature.

The infectivity and haemagglutinating properties of the virus were almost completely preserved in the presence of either concentration of serum albumin, and only a 50% loss was obtained with the preparations stored in sucrose or glucose solutions. Glycerol, particularly at the higher concentration, caused a marked loss in infectivity, whereas freeze-drying resulted in an almost complete loss of biological properties. The results with Earle's medium were variable, but usually no more than 25% of infectivity was retained. The method of preservation now in use is to prepare ampoules of a clear suspension of the virus containing about 40 μg. of RNA/ml. in a sterile solution of 0·02 M-phosphate buffer containing 0·2% of bovine serum albumin or 10% (w/v) of glucose. The ampoules are stored at −70° and diluted with 3 vol. of Earle's medium after thawing before use. Storage under these conditions has resulted in excellent preservation of infectivity.

Ratio of infectivity to haemagglutinin during purification

In three preparations F44, F64 and F66 (cf. Table 5) the haemagglutinin and plaque-forming activities were estimated on the crude preparation as well as on the purified virus. The ratios of plaque-forming units to haemagglutinin units were $1·2 \times 10^5$, $1·2 \times 10^5$ and $1·9 \times 10^5$ before purification and $2·5 \times 10^5$, $1·1 \times 10^5$ and $1·2 \times 10^5$ after purification.

Precipitating antibody against encephalomyocarditis virus

Several attempts were made to immunize rabbits against EMC virus by intramuscular and intravenous injection of virus but no precipitating antibody was formed. In a further series of experiments rabbits were injected intracerebrally. Five animals received 1×10^7 plaque-forming units each and survived with no apparent symptoms. After 17 days these animals each received an intravenous injection of 1×10^{10} plaque-forming units and after 120 days a further 1×10^{11} units of purified virus. Trial bleedings were made 10, 13, 16 and 20 days after the last injection, and a steady rise in the titre of precipitating antibody was observed. In further experiments it was found that the best antiserum was produced by two intracerebral injections followed by two intravenous injections. In this way antisera were produced which precipitated virus at 1/640. Non-specific antibody against ascites-tumour-cell protein could not be detected. Several animals were injected with the microsomal fraction of tumour cells and failed to develop antibodies against this protein.

Agar-gel precipitation of encephalomyocarditis virus

Ouchterlony plates were prepared by the method of Pereira, Pereira & Allison (1959). The circumferential cups were filled with crude virus-culture supernatant, purified virus or an ultrasonic homogenate of uninfected cells. To one set of plates antiserum was added immediately (centre cup) and the plates were stored at 0°. The other set of plates were left 3 days at 0° without antiserum and the circumferential cups were refilled with 0·9% sodium chloride. After another 3 days this process was repeated and the centre cup was then filled with antiserum. The first set of plates showed slight precipitation on the edge of the cup containing purified virus but no precipitation elsewhere. This result is explicable since the large size of the virus will result in extremely slow diffusion through agar. The second set of plates showed clearly defined precipitation zones around the cups containing purified virus but no lines around either the cell protein cups or the crude virus-culture supernatant. From these results it was evident that intracerebral injection followed by intravenous injection had resulted in the production of a rather specific anti-EMC serum.

DISCUSSION

The smaller animal viruses are rather stable nucleoprotein complexes and their purification presents much the same problems as are presented by any similar group of macromolecules. The main impediment to progress has probably been inability to obtain virus in sufficient quantity and in this respect we were fortunate in that the methods of Hoskins & Sanders (1957) for growth of EMC virus in Krebs cells could be scaled upwards without difficulty. The problems involved in purification and in identification of a particular type of particle as an infective unit have been fully reviewed by Sharp (1953) and need not be repeated here. The conclusion that our crystalline preparation represents pure EMC virus depends on the several complementary lines of evidence set out in our Results. No one line of evidence is conclusive when considered alone but taken together, they amount to a fairly rigid proof of our assumption that the crystalline material is indeed pure EMC virus.

The ratio of about 1:10 between infective units and particle counts is about the same as that achieved with other animal virus preparations. The problem of the relationship between particle count and infectivity has been fully discussed by Isaacs (1957). Although the total count and the infective particle count are roughly equal for some bacteriophages (Luria, Williams & Backus, 1951), the failure to reach a 1:1 ratio does not necessarily indicate that the preparation is impure. There is no precise knowledge of the number of particles required to infect a single cell and in the absence of this knowledge no definite conclusion can be reached regarding the infectivity of individual particles. The analytical ultracentrifuge and the electron microscope have indicated that our preparation consists of physically uniform particles and ready crystallization of the purified virus reinforces this conclusion.

In size, shape, protein:nucleic acid ratio, amino acid composition and purine–pyrimidine composition, EMC virus resembles poliomyelitis virus. Yčas (1960) has suggested that the base composition of the RNA of a virus may be calculated from the amino acid composition of its protein. Such a calculation suggests that the base ratios should be guanine 23·3, adenine 29·7, cytosine 22·0, uracil 25·0. These calculated figures agree moderately well with the analytical figures (Table 5) except for adenine. Unfortunately we have not been able to prepare sufficient crystalline virus to permit repetitive analyses and determination of the standard error, but we consider it unlikely that the analytical figures for the amino acids would be in error to a sufficient degree to account for the discrepancy. This is, however, obviously a point upon which additional information is required.

Since the ratio of haemagglutinin to infectivity remained constant throughout the purification we conclude that the haemagglutinin is the protein of the virus. This is an important conclusion since it

has enabled us to follow synthesis of virus protein by following the incorporation of radioactive amino acids into haemagglutinin.

SUMMARY

1. A method for large-scale production of encephalomyocarditis virus has been developed.

2. The behaviour of the virus on columns of diethylaminoethylcellulose, ECTEOLA-cellulose and calcium phosphate has been studied.

3. Crystalline virus has been obtained from effluents after chromatography on calcium phosphate columns.

4. The virus has been shown to be a ribonucleoprotein containing about 30% of ribonucleic acid.

5. Electron-microscope photographs show that the virus is a spheroid with a diameter of about 27 mμ (dry).

6. Particle counts indicate that about 10% of the particles in a crystalline preparation are infective (as shown by plaque counts). This compares very favourably with crystalline preparations of poliomyelitis virus.

7. The base composition of the viral ribonucleic acid has been determined and also the amino acid composition of the viral protein.

8. Virus haemagglutinin is associated with and is purified along with the virus. It is concluded that the haemagglutinin is the virus protein.

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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

3. METHODS FOR THE DISRUPTION OF KREBS II MOUSE-ASCITES-TUMOUR CELLS

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We have reported on the changes in nucleic acid and protein metabolism in Krebs II ascites-tumour cells during the growth of encephalomyocarditis virus in this cell (Martin, Malec, Sved & Work, 1961). For further work, it was decided to study the metabolic changes in different subcellular fractions of the Krebs cell. Before this could be done, however, methods had to be devised for the rupture of Krebs cells and for the quantitative separation of the subcellular particles.

The Krebs II mouse carcinoma in the ascitic form (Klein & Klein, 1951) is mechanically an exceptionally tough cell and is quite undamaged by homogenizing in a Potter–Elvehjem homogenizer with either sucrose or inorganic buffers. Our objective was to disrupt with as little damage as possible to subcellular components. Damage was assessed by three independent criteria: firstly, the microscopic appearance of the products, secondly, the distribution of deoxyribonucleic acid, ribonucleic acid and protein in separated nuclei, mitochondria, microsomes and cell sap, and thirdly, the capacity of the microsomal fraction to incorporate amino acids into protein. The third criterion is admittedly selective and arbitrary but it was of special interest in our investigations on virus biosynthesis.

METHODS

Homogenizers. Two types of homogenizer were used. The Potter–Elvehjem homogenizer consisted of a 15 ml. capacity, round-bottomed centrifuge tube fitted with a polytetrafluoroethylene (Teflon) pestle, with a clearance of about 0·2 mm. between pestle and the glass tube. The second homogenizer was made of glass according to the instructions of Dounce (1955) and will be referred to throughout as the Dounce homogenizer. In this homogenizer the clearance between the glass ball and the tube wall was only about 0·03 mm. on a 1·2 cm. diameter precision bore glass tube.

Ultrasonics oscillator. A Mullard laminated nickel magnetostriction transducer was brazed to a stainless-steel cup of 2 cm. diameter and 7·5 cm. length. This was coupled to an oscillator with an output of 200 w and tuned to 25 kcyc./sec. The apparatus is similar to that described by Davies (1959). When in use, the steel cup was surrounded by crushed ice and partially filled with ice–water. The cell suspension (2 ml.) in a thin-walled plastic tube (Spinco tube) was immersed in the ice–water.

Analyses of cell fractions. The methods were those described by Martin *et al.* (1961). Where necessary, the preferred method is indicated in the appropriate Table.

Assay for radioactivity. Proteins were isolated, estimated and assayed for radioactivity as described by Martin *et al.* (1961). Randomly labelled [^{14}C]amino acids (The Radiochemical Centre, Amersham, Bucks) were used throughout.

Special chemicals. Phosphoenolpyruvate, 3-phosphoglycerate and pyruvate kinase were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany.

Assay of succinoxidase. The succinoxidase activity of various cell fractions was measured in a Warburg apparatus according to the method of Schneider & Potter (1943).

RESULTS

Ultrasonic oscillator

Krebs II mouse-ascites cells were washed in phosphate-buffered saline as described by Martin *et al.* (1961), packed by centrifuging and resuspended in the media listed in Table 1. The suspensions were exposed to the sonic oscillator for various times and samples were withdrawn for microscopic examination in nigrosin–potassium chloride (Martin *et al.* 1961). With this dye, nuclei appear as uniformly stained disks free of cytoplasm, while undamaged cells are unstained. It was clear from the results, that the time of exposure to ultrasonics was highly critical and that magnesium chloride had a considerable stabilizing influence; indeed, preparations in pure sucrose were gelatinous, indicating that the DNA had been released from the nuclei.

Although it was evident that ultrasonics were unlikely to give a preparation containing 100% undamaged nuclei, the sucrose–magnesium chloride–tris mixture looked promising and was further investigated. Washed ascites cells were suspended in this medium, disintegrated, centrifuged and the precipitate was discarded. The supernatant was incubated with [^{14}C]valine under the conditions

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given in Table 2 and the radioactivity of the protein was then measured. The results indicated that, although ultrasonics tended to break nuclei, the microsome–cell sap fraction was active in protein biosynthesis. However, the degree of nuclear disruption was difficult to control and alternative methods of cell disruption were therefore investigated.

Table 1. *Disintegration of Krebs II mouse-ascites-tumour cells by ultrasonics*

The percentage of morphologically intact free nuclei was estimated by inspection and counting in a Neubauer cell after dilution with nigrosin (0·2%) in KCl (0·3M). The method of disintegration is described in the text. Tris–HCl buffer, pH 7·5, is referred to as tris.

Suspending medium (1×10^8 cells/ml.)	Duration of exposure to ultrasonics (sec.)	Percentage undamaged free nuclei
0·25M-Sucrose	20	None
	30	None
	40	20
	50	60
	60	Nearly all nuclei broken
0·25M-Sucrose, 0·05M-tris	40	20
	50	60
	60	Nearly all nuclei broken
0·25M-Sucrose, 5 mM-$MgCl_2$	50	None
	60	5
	75	15
0·25M-Sucrose, 0·05M-tris, 5 mM-$MgCl_2$	40	10
	50	50
	60	70 (some broken nuclei)

Table 2. *Incorporation of [^{14}C]valine into the microsome–cell sap fraction of Krebs II ascites-tumour cells after disintegration by ultrasonics*

Washed cells (3×10^8) were suspended in 2 ml. of medium with components at the following concentrations: sucrose, 0·25M; KCl, 0·025M; $MgCl_2$, 5 mM; tris buffer, pH 7·5, 0·05M. After disintegration by ultrasonics (50 sec.) the homogenate was centrifuged (10 000*g* for 10 min.) and the supernatant incubated for 30 min. at 37° with 2 μc of [^{14}C]valine and the energy source indicated. PGA, Potassium salt of 3-phosphoglyceric acid; ATP, potassium salt of adenosine triphosphate.

Energy source	Radioactivity of protein* (μmc/g.)
ATP, 10 μmoles	14
ATP, 10 μmoles + PGA, 15 μmoles	76
ATP, 5 μmoles + PGA, 33 μmoles	115
PGA, 33 μmoles	294

* The microsomes were not isolated after incubation and this material was a mixture of cell-sap protein and microsomal protein (cf., however, Table 3).

Dounce homogenizer

When Krebs II mouse-ascites-tumour cells were suspended in sucrose–calcium chloride (0·25M–5 mM) and homogenized as described by Dounce (1955) the preparation had excellent morphology with almost 100% breakage of the cells and few damaged nuclei but the microsome–cell sap fraction did not incorporate [^{14}C]valine. When magnesium chloride (0·01M) was substituted for calcium chloride excellent incorporation of [^{14}C]-valine into the microsome–cell sap fraction was obtained and the free nuclei were clean and appeared to be undamaged. About 70% cell breakage was obtained before any significant number of damaged nuclei appeared. The activity in protein synthesis was closely similar to that of ultrasonic microsomes prepared from another sample of the same batch of cells (Table 3). In this experiment, the addition of soluble RNA did not greatly increase the rate of incorporation but did prolong the period of incorporation.

Analytical composition of subfractions of Dounce homogenate

Freshly collected ascites-tumour cells were homogenized in sucrose–magnesium chloride medium and separated by differential centrifuging into four fractions consisting of nuclei, mitochondria, microsomes and cell sap. Each fraction was analysed for total nucleic acid, DNA and protein (Table 4). In this experiment, an attempt was made to carry the homogenizing treatment to the point where no whole cells remained. Although it was easy to obtain 70% disruption, 100% disruption could not be achieved without damage to nuclei as indicated by appearance of DNA in fractions other than the nuclear fraction.

Disruption of cells by homogenizer after osmotic shock

Since neither ultrasonics nor the high shear of a Dounce homogenizer produced complete cell disruption without damage to nuclei, it was decided to try the combined effect of osmotic shock and either the Dounce- or the Potter-type homogenizer. Conditions were worked out for the successful application of either homogenizer but the conditions were quite different for the two types of instrument. As each had advantages, they will be described separately. Severe osmotic shock coupled with gentle homogenizing in the Potter homogenizer gave a preparation that was morphologically and analytically most satisfactory, but mild osmotic shock followed by severe homogenization in the Dounce homogenizer gave a preparation that was more effective in tests of capacity for protein biosynthesis.

Potter homogenizer

It was noted (Table 1) that Mg^{2+} ions have a considerable stabilizing effect on Krebs cells and in any buffer that contained Mg^{2+} ions the cells were completely resistant to the Potter homogenizer. If, however, cells were washed in buffer free of Ca^{2+} or Mg^{2+} ions (solution *A* of Martin *et al.* 1961, diluted with one-quarter of its own volume of water), they became less stable and could then be disrupted by a combination of osmotic shock and gentle homogenizing. The most successful method is given below.

Table 3. *Incorporation of* [^{14}C]*valine into microsomal protein during incubation with cell sap and soluble ribonucleic acid*

Microsome–cell sap mixtures were prepared by ultrasonic disintegration as in Table 2 or by the Dounce homogenizer as described in the text. Each flask contained microsomes and cell sap from 2×10^8 cells, 2 μc of [^{14}C]valine and 20 μmoles of potassium phosphoglycerate in a final volume of 3·7 ml. Other components were present in the following final concentrations: sucrose, 0·25 M; tris buffer, pH 7·5, 0·05 M; $MgCl_2$, 5 mM. After the stated period of incubation excess of [^{12}C]valine in sucrose (0·25 M) was added and the microsomes were spun down at 100 000*g*. Protein was isolated in the usual way.

Components	Incubation time (min.)	Radioactivity of microsomal protein (μmc/g.)
Dounce microsomes + cell sap	0	24
	20	924
	40	840
Sonic microsomes + cell sap	0	23
	20	888
	40	880
Dounce microsomes + cell sap + soluble RNA (1·0 mg.)	20	960
	40	1230*

* Soluble RNA was prepared from Krebs cells by the method described by Allen, Glassman & Schweet (1960).

Freshly collected Krebs ascites-tumour cells were washed twice with phosphate-buffered saline (Martin *et al.* 1961) and once with diluted solution *A*. The cells were resuspended in sucrose–potassium chloride (0·125 M–0·075 M) and counted. About 3×10^8 cells were spun at 2200*g* for 5 min. at 0° and the cell volume (V_1) was noted. The supernatant was replaced by ice-cold water ($5V_1$) and the cells were resuspended. The suspension was immediately recentrifuged at 0° (2200*g* for 3 min.) and the cell volume (V_2) noted. The supernatant was discarded and replaced by ice-cold water ($11V_1 - V_2$). The cells were resuspended by gentle hand homogenizing and, after 5 min. at 0°, sucrose (1·5 M) was added to a final concentration of 0·1 M. After a further 10 min. at 0° the suspension was vigorously homogenized with the Potter homogenizer (3000 rev./min. for 15 sec.) and sucrose–potassium chloride (0·75 M–0·5 M) was immediately added to a final potassium chloride concentration of 0·1 M. This method gave about 85% cell rupture and clean undamaged nuclei.

Isolation of subcellular fractions

The whole cells and nuclei were spun down together, resuspended in sucrose (0·3 M) containing EDTA (sodium salt; 0·04 M; adjusted to pH 7·3 with N-potassium hydroxide), washed once with this mixture, centrifuged at 2200*g* for 5 min., and resuspended in ice-cold water ($8V_1$) by gentle hand homogenizing. Sucrose (1·5 M) was added at once to a final concentration of 0·1 M and after 10 min. at 0° the preparation was homogenized in the Potter homogenizer (3000 rev./min. for 15 sec.). Sucrose–potassium chloride (0·75 M–0·5 M) was immediately added as before and the nuclei were collected by spinning at 450*g* (5 min.). The nuclei were now hardly contaminated by whole cells. The overall yield of nuclei was about 85% of the number of cells taken.

Table 4. *Distribution of deoxyribonucleic acid, total nucleic acid and protein in subfractions of an ascites-tumour-cell homogenate prepared in a Dounce homogenizer with sucrose–magnesium chloride*

Washed tumour cells (2×10^8) were suspended in 3 ml. of sucrose–$MgCl_2$ (0·25 M–0·01 M), cooled to 0°, and homogenized with about 200 strokes of a Dounce homogenizer as described in the text. The homogenate was spun at 700*g* for 5 min. The pellet was resuspended in 20 ml. of sucrose (0·25 M) and recentrifuged at 700*g*. The combined supernatants were spun at 700*g* for 5 min. and the pellet (intermediate) was analysed separately. The sucrose supernatants were recentrifuged first at 6000*g* for 20 min. (mitochondria) and then at 100 000*g* for 60 min. (microsomes). All fractions were resuspended in 0·9% NaCl and, together with the final supernatant, were treated with $HClO_4$ (final concentration 0·5 M). Total nucleic acid, DNA and protein were estimated as described by Martin, Malec, Sved & Work (1961).

Fractions	Total nucleic acid (μg. of P/10^8 cells)	DNA (μg. of P/10^8 cells)	Protein (mg./10^8 cells)
Nuclei + whole cells	214	193	4·9
Intermediate	5·5	3·8	0·21
Mitochondria	35·4	7·2	1·96
Microsomes	114	0·48	2·16
Cell sap	31·6	0	5·40

Table 5. *Distribution of deoxyribonucleic acid, ribonucleic acid, protein and succinoxidase in subcellular fractions obtained from Krebs II ascites-tumour cells by osmotic shock*

The methods of disruption and analyses are described in the text. The fraction referred to as intermediate is material which spun down at 700*g* after the nuclei had been removed from the main homogenate. Percentage recovery in each case is based on analysis of the whole lysate after the initial disruption.

	Protein		RNA		DNA		Succinoxidase	
Fraction	mg./10^8 cells	%	μg. of P/ 10^8 cells	%	μg. of P/ 10^8 cells	%	μl. of O_2/ 10^8 cells/hr.	%
Nuclei	1·43	8·7	26·7	11·7	193	100	6	0·7
Intermediate	0·16	0·97	2·9	1·2	0·8	0·5	23	2·5
Mitochondria	2·27	13·8	35·8	15·7	0	0	937	102
Microsomes	2·72	16·5	139	61·1	0	0	0	0
Cell sap	5·52	33·5	26·4	11·6	0	0	0	0
Percentage recovery	—	73·5	—	102·3	—	100·5	—	105·2

Mitochondria were separated from the combined cytoplasmic fractions by centrifuging (10 000*g*), washed by resuspension in sucrose–potassium chloride (0·25 M–0·1 M) and recentrifuged.

Microsomes were collected at 100 000*g* for 1 hr. in a Spinco preparative centrifuge. They were not washed. Cell sap was taken to be the material that did not spin down at this speed.

Distribution of succinoxidase, deoxyribonucleic acid, ribonucleic acid and protein in subcellular fractions prepared by osmotic shock

As an index of the effectiveness of the above method of cell rupture the different fractions were analysed for DNA, RNA and protein by the methods used earlier (Martin *et al.* 1961), and in addition all fractions were assayed for succinoxidase activity; the results are given in Table 5. Since virtually all the DNA was found in the nuclear fraction and all the succinoxidase in the mitochondrial fraction the method is apparently satisfactory. This conclusion is reinforced by the presence of over 60% of the RNA in the microsomal fraction. The amount of RNA that has been leached out of the nuclei cannot be large since the ratios of RNA to DNA and of protein to DNA are within the range usually found for nuclei prepared by non-aqueous techniques (Allfrey, Mirsky & Stern, 1955). Also, the value for RNA in the cell sap is low. It is reasonable to suppose that if any significant quantity of RNA had been leached out of the nuclei it would have appeared in this fraction. The rather low recovery of protein in these experiments (Table 5) is probably explained by the physical characteristics of the nuclear proteins. The method of isolation of protein (Martin *et al.* 1961) involved extraction of the nuclei with hot perchloric acid. Further losses occurred when the acid-extracted nuclei were extracted with alkali since the nuclei contained material (probably glycoprotein) insoluble in alkali (cf. Dallam, 1954). The almost complete absence of succinoxidase from the nuclear fraction indicates that it is free from cytoplasmic or whole-cell contamination, and the complete absence of this enzyme complex from the microsomal fraction indicates that the mitochondria have not been disrupted.

Protein biosynthesis by osmotic microsomes

The obvious analytical advantages of osmotic shock combined with the Potter homogenizer are accompanied by certain disadvantages with respect to protein biosynthesis in cell-free preparations. First, the cell sap is too dilute to be used as a suspending medium for the microsomes, and, secondly, even when concentrated cell sap is prepared by other methods and combined with these microsomes, the rate of incorporation is lower than that achieved with the Dounce homogenate. In an attempt to overcome the disadvantages of both methods further experiments were made in which mild osmotic shock was combined with the use of a Dounce homogenizer. The conditions which were most successful in producing a high disruption rate, a limited degree of nuclear damage and a microsome fraction with a high capacity for amino acid incorporation are set out in Table 6. This method is preferable to the original Dounce method or the magnesium chloride–sucrose Dounce method in that it is very much more rapid but it does not give such effective separation between subcellular fractions as can be obtained by the more rigorous double-osmotic-shock method. Analyses of the various subcellular fractions obtained by this combination of osmotic shock and the Dounce homogenizer are given in Table 7. Although nearly all the DNA was found in the nuclear fraction, the proportion of protein found in this fraction was too high presumably because the method did not produce nuclei free from adherent cytoplasm. Nevertheless, this method was adopted as the most suitable for rapid preparation of a microsome–cell sap system capable of protein biosynthesis.

It will be noticed in comparing Tables 4, 5 and 7 that the total DNA per 10^8 cells varies over the range 193–228 μg. of DNA phosphorus. This

variation is within the range of the sampling and counting error in the Neubauer cell and does not necessarily indicate variability in the DNA content of successive Krebs cell preparations. It might be permissible to adjust the figures in all four tables to a common DNA value but we have insufficient evidence to justify this.

Osmotic shock potentiated by glycerol

Since the methods of osmotic shock described above resulted in a very dilute solution of cell sap, a further attempt was made to overcome this disadvantage by potentiating the osmotic shock with the aid of glycerol. It is known from the work of Lovelock (1954) and others that glycerol passes freely across the membranes of mammalian cells. Krebs II ascites-tumour cells were therefore washed in Ca^{2+}- and Mg^{2+}-free phosphate buffer and incubated with 15% glycerol at various temperatures and for various times. In the early stages of incubation the cell wall was heavily reticulated and the cell size greatly reduced but after 30 min. at 26° or 5 min. at 37° the cells had returned to normal size. The cells were spun down and submitted to osmotic shock by resuspension in water.

The treatment gave effective disruption but the separation between cell components was not as clean as with the more elaborate double-osmotic-shock method. The percentage of protein in the nuclear fraction was too high and that in the mitochondrial and microsomal fractions too low. These results suggest that cytoplasmic debris had clung to the nuclei. Attempts to improve the method were not pursued since microsomes prepared by glycerol-potentiated osmotic shock had only about one-eighth the amino acid-incorporating activity of Dounce–magnesium chloride microsomes in the usual test with [^{14}C]valine.

Table 6. *Incorporation of* [^{14}C]*valine into microsome protein by Krebs II ascites-tumour cells disrupted in a Dounce homogenizer after mild osmotic shock*

Cells were washed in phosphate-buffered saline, centrifuged at 0° and 2300*g* for 3 min. and the packed cell volume (*V*) was noted. Cells were resuspended in 0·01 M-$MgCl_2$ (3*V*) at 0° and after 2 min. they were disrupted by 10–20 strokes of the Dounce homogenizer. Sucrose (1·5 M) was added immediately to a final concentration of 0·25 M and the homogenate spun at 6000*g* for 15 min. The supernatant (2 ml.) was incubated with [^{14}C]valine (2 μc) together with potassium phosphoglycerate (20 μmoles), salts and buffer as indicated in Table 3. A second sample of the same batch of cells was disrupted in sucrose–$MgCl_2$ as described in Table 3.

Medium for disruption	Incubation time (min.)	Radioactivity of protein (μmc/g.)
$MgCl_2$	0	29
Sucrose–$MgCl_2$	0	30
$MgCl_2$	40	1150
Sucrose–$MgCl_2$	40	1200

DISCUSSION

The results presented in this paper emphasize the difficulty encountered in the preparation of undamaged nuclei, mitochondria and microsomes from the Krebs II ascites-tumour cell. This cell appears to be exceptionally robust, and methods which are successful with the Ehrlich ascites-tumour cell (Littlefield & Keller, 1957) disrupt only a small percentage of Krebs cells. The use of the Dounce homogenizer, combined with a form of osmotic shock, has been described by Hawtrey & Silk (1960) and by Sauer, Martin & Stotz (1960) for the rupture of Ehrlich cells. It is difficult to judge the efficacy of either of these methods, however, since neither group of authors has published a full analysis of their subcellular fractions.

McComb & Yushok (1959) have successfully disrupted Krebs cells in a mixture of sucrose and EDTA. This method obviously depends on the complete removal of Ca^{2+} and Mg^{2+} ions, but cells disrupted in this way have given, in our hands, microsome–cell sap preparations that are incapable of protein biosynthesis.

The preparation of clean nuclei from the crude 700*g* precipitate obtained from the first lysis in the double-osmotic-shock method involves the selective rupture of undamaged cells, previously treated with an agent forming a complex with a bivalent cation, when suspended in dilute sucrose solutions.

Table 7. *Distribution of deoxyribonucleic acid, total nucleic acid and protein in subfractions of an ascites-cell homogenate prepared with a Dounce homogenizer after mild osmotic shock*

Cells (3×10^8) were disrupted in 0·01 M-$MgCl_2$ as described in Table 6. Sucrose (1·5 M) was added immediately to a final concentration of 0·25 M and the suspension was rehomogenized rather gently. The subcellular fractions were separated as in Table 4.

Fraction	Total nucleic acid (μg. of P/10^8 cells)	DNA (μg. of P/10^8 cells)	Protein (mg./10^8 cells)
Nuclei + whole cells	250	228	6·6
Intermediate	4·2	1·9	0·13
Mitochondria	22·2	1·7	1·4
Microsomes	124	0·95	2·0
Cell sap	34·6	0·0	6·3

Under these circumstances nuclei swell, but do not burst, while whole cells are rapidly disrupted. This process was relatively independent of sucrose concentration and was observed to occur in both 0·1 M- and 0·8 M-sucrose solutions. Both Krebs II and Ehrlich ascites-tumour cells, but not isolated single rat-liver cells (Branster & Morton, 1957), behaved in this manner. The reversible swelling and shrinking of isolated nuclei when suspended in concentrated sucrose solutions followed by the addition of salts has been frequently observed (Anderson & Wilbur, 1952; Philpot & Stanier, 1956; Battin, 1959) but the rupture of ascites-tumour cells under similar circumstances has hitherto not been reported.

It is notorious that methods which give, in good yield, undamaged nuclei are usually less suitable for the isolation of other cell components (Allfrey, 1959), and this difficulty is accentuated when the cell membrane is unusually robust as it is in the Krebs cell. The success of the double-osmotic-shock method described here depends upon the exclusion of Ca^{2+} and Mg^{2+} ions during the swelling process and the addition of potassium chloride immediately after disruption.

Since the method gives quantitative recovery of RNA and DNA and clean separation between nuclei, mitochondria and microsomes, it has been used by us in further studies on the localization of the sites of metabolic change during the course of growth of encephalomyocarditis virus in Krebs II cells (Martin & Work, 1961). It has also been used extensively in the localization of the site of formation of infective RNA within these cells (Bellett & Burness, 1960). The method is not, however, completely satisfactory where the objective is the preparation of a microsome–cell sap mixture capable of protein biosynthesis, and for this purpose we have preferred mild osmotic shock in magnesium chloride and disruption with the Dounce homogenizer.

SUMMARY

1. Krebs II ascites-tumour cells can be ruptured by ultrasonics, by osmotic shock or by the high shear of a Dounce homogenizer.

2. Cells ruptured in a Ca^{2+}- and Mg^{2+}-free medium by a combination of osmotic shock and a low-shear homogenizer gave high yields of undamaged nuclei, mitochondria and microsomes.

3. Nuclei prepared by the osmotic method and separated from contaminating whole cells by a novel procedure, were free from succinoxidase and had low RNA:DNA ratio.

4. Microsomes prepared by osmotic-shock methods in the absence of Mg^{2+} ions were less active in incorporating radioactive valine than were microsomes prepared in the presence of Mg^{2+} ions by ultrasonics or by a high-shear homogenizer.

5. The Krebs II ascites-tumour cell is unusually robust and cannot be ruptured by methods which are adequate for other types of cell.

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The Oxidation of Protein Thiol Groups by Iodate, Bromate and Persulphate

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The oxidation of some simple thiols by oxidizing agents known to act as improvers in bread manufacture has been investigated (Hird & Yates, 1961). It was shown that when cysteine was oxidized the major product was the disulphide cystine, but that small amounts of cysteinesulphinic acid and cysteinesulphonic acid were minor products (approx. 10%). Cystine, however, was not oxidized by these reagents under the conditions used. It has also been shown that the thiol groups of gluten formed by reduction with sodium borohydride were oxidized (Frater, Hird, Moss & Yates, 1960), but the nature of the oxidation products was not investigated.

The work described in the present paper attempts to define more precisely the action of iodate, bromate and persulphate on the thiol groups of certain protein preparations, particularly those from flour. In the flour being investigated the disulphide content of the total proteins is approximately ten times that of the thiol content when considered on the basis of sulphur atoms (Frater *et al.* 1960). Because of the low thiol content compared with the –S–S– groups already present, accurate estimation of the yield of disulphide when thiol groups are oxidized is difficult. Further, isolation of the proteins is accompanied by atmospheric oxidation of thiol and it has therefore been necessary to reduce the protein preparations with sodium borohydride to obtain preparations with sufficient thiol content for subsequent experiments.

The oxidation of thiolated gelatin by ferricyanide has been studied by Benesch & Benesch (1958) and this substance, because of its high thiol content and suitable solubility properties, has been included in the present investigation.

MATERIALS AND METHODS

The gluten was a commercial sample prepared by acetic acid extraction of flour and was presented by Barretts Ltd., Melbourne. The water-soluble proteins were prepared by extraction of 1 kg. of flour (Victorian FAQ) with 10 l. of water for 1 hr., followed by freeze-drying of the extract. The thiolated gelatin was a commercial sample obtained from Schwartz Laboratories Inc., Mount Vernon, N.Y. As obtained it contained approx. 16 moles of thiol groups/10^5 g. of protein.

Sodium borohydride was obtained from Metal Hydrides Inc., Beverley, Mass., U.S.A.

Estimation of thiol and disulphide groups

Thiol and disulphide groups in the proteins were determined amperometically by estimation of excess of methyl mercuric iodide added to the system at the end of the reaction time. The titration was done at $-0{\cdot}7$ v with a dropping mercury electrode with Ag–AgCl reference electrode. A sample of the reaction mixture (10 ml.) was removed at the appropriate time interval, and added to a known excess of methyl mercuric iodide to stop the oxidation reaction. A portion (10 ml.) of aq. 0·25 M-NH_3 soln.–NH_4Cl buffer, 8 M with respect to urea, at pH 9·0, was then added. The temperature of the reaction vessel was kept at $25 \pm 0{\cdot}05°$. A separate base line was determined in the absence of mercurial for each sample analysed. The current relative to this at $-0{\cdot}7$ v was then measured on the experimental sample, and serial additions of 0·2 ml. of methyl mercuric iodide, 2 mM in 25% dimethylformamide, were added, and the current was determined in each case. The number of thiol groups present was calculated by extrapolation to zero current.

For the estimation of disulphide, the buffer was made 0·2 M with respect to Na_2SO_3, which raised the pH from 9·0 to 9·25 and the extra thiol obtained was taken to be equal to the disulphide in the sample, as in the presence of the mercurial the reaction goes to completion (Leach, 1960). Before estimation of disulphide, the protein was dispersed in a tightly fitting Potter–Elvehjem type homogenizer in the presence of the sulphite and excess of methyl mercuric iodide. These manipulations were done at room temperature (18–20°). The total reaction time with the mercurial was 10–15 min., including the gassing of the reaction vessel. This is in excess of that required for the reaction to go to completion (see also Leach, 1960). Under these conditions cystine disulphoxide is not estimated as disulphide.

The estimation of thiol groups with methyl mercuric iodide involves an assumption of absolute specificity of this reagent. To check this point several samples of gluten were analysed for cysteine + cystine sulphur by the above-described procedure and by the differential-oxidation method of Lugg (1938). For three separate samples the results were 13·0, 12·4 and 13·6 moles of S/10^5 g. of protein when estimated polarographically and 13·8, 14·1 and

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Table 1. *Formation of disulphide bonds on oxidation of reduced gluten*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μmoles/100 mg. of protein.

	Iodate			Bromate			Persulphate		
Time	–SH	–S–S–	Total S (–SH + –S–S–)	–SH	–S–S–	Total S (–SH + –S–S–)	–SH	–S–S–	Total S (–SH + –S–S–)
0	9·7	3·1	15·9	9·7	3·1	15·9	9·7	3·1	15·9
30 min.	0	6·5	13·0	—	—	—	—	—	—
24 hr.	—	—	—	1·5	5·0	11·5	0	6·8	13·6

15·3 moles of S/10^5 g. of protein respectively when estimated by Lugg's procedure. There is therefore reasonable agreement between the two methods.

Preparation of reduced proteins

Gluten. Reduced gluten was prepared by dispersing as finely as possible 2 g. in 200 ml. of water in the Potter–Elvehjem homogenizer, the final suspension being made 0·1 M with respect to sodium borohydride. Frothing caused by liberation of hydrogen was kept at controllable levels by the use of octanol and reduction was carried out for 30 min. at 50°. Treatment for longer periods was found to result in a decrease in the number of thiol groups. The addition of urea to the system to open up the protein structure was impracticable as it caused difficulties in the isolation of the reduced protein.

After reduction the system was cooled to 0°, brought to pH 6 with HCl and stirred for 30 min. at 0°. This procedure has the dual effect of destroying the excess of borohydride and precipitating the reduced gluten. The protein was isolated by centrifuging, washed twice with 5 mM-EDTA and freeze-dried. Of the original gluten 50–60% was obtained in the reduced state. On reduction the thiol content rose from 1·2 to approx. 11 moles of thiol/10^5 g. of protein, depending on the preparation.

Water-soluble proteins. With the water-soluble proteins, the reducing system contained 0·2 g. of protein/100 ml. of water. The reduction was carried out at 50° for 30 min. in 0·05 M-sodium borohydride. After this time an equal volume of 0·5 M-citrate buffer, pH 6, was added and the solution stirred for 30 min. as before. The solution of reduced water-soluble proteins was used immediately after preparation and thus contained metaborate, the decomposition product of the borohydride. It was found to be impossible to reduce completely the water-soluble proteins, a maximum of six thiol groups being obtained out of a possible 12·6.

Oxidation of reduced protein. A suitable quantity of protein (approx. 1 mg./ml.) was dissolved or finely dispersed (for reduced gluten) in 0·5 M-citrate buffer, pH 6·0. After addition of oxidizing agent the system was gently shaken in a constant-temperature bath throughout the period of reaction. The reaction was carried out at 28° under oxygen-free nitrogen.

After the appropriate time, 10 ml. of the sample was pipetted into 10 ml. of the appropriate analytical system containing the excess of methyl mercuric iodide and the thiol and disulphide content were estimated.

Oxidation of flour. The oxidation of flour was carried out in an aqueous suspension consisting of 20 ml. of water and 1 g. of flour. The oxidizing agents were added so that the molar ratio of thiol/oxidizing agent was 1/1. After the appropriate reaction time, 1 ml. of aq. NH_3 soln.–NH_4Cl buffer (2 M) was added to the system so that the final pH was 9·0, and the thiol content estimated.

Table 2. *Molar ratio thiol/oxidizing agent at the equivalence point with reduced gluten*

Concentration of protein: 10 mg./10 ml. Iodate was added to oxidize an increasing percentage of thiol groups. Reaction time: 15 min. Percentage of thiol groups oxidized is given in parentheses. For other conditions see text.

Initial thiol content of system (μmoles)	Iodate added (μmole)	Thiol oxidized (μmole)	Thiol groups oxidized/ oxidizing agent added
1·18	0·056	0·20 (18%)	3·7
1·18	0·16	0·72 (61%)	4·5
1·18	0·23	0·98 (83%)	4·3

RESULTS

Table 1 shows the thiol and disulphide distribution of reduced gluten after oxidation by the reagents used. Oxidation by iodate brings about a definite and an immediate decrease of thiol groups and an increase in the number of disulphide bonds. With bromate and persulphate there is also an increase in the number of disulphide bonds, but the oxidation by these reagents is much slower than with iodate. It can also be seen that there is a decrease in the amount of sulphur recoverable as cysteine plus cystine after oxidation. This is consistent with the formation of higher oxidation products found with cysteine (Hird & Yates, 1961) and also with the experimentally determined molar ratio thiol/iodate of 3·7–4·4 at the equivalence point (Table 2). In these experiments iodate has been used for the convenience of its rapidity of action. It can be seen, too, that the ratio does not change appreciably with the different percentages of thiol groups oxidized (see also Table 6).

Table 3 shows that the thiol groups of the reduced water-soluble proteins are oxidized but less completely than those of reduced gluten (Table 1) and thiolated gelatin (Table 5). This resistance to oxidation parallels the resistance of these prepara-

tions to reduction. The number of thiol groups disappearing is too small for any accurate estimation of the disulphide formed.

As Table 4 shows, 50% or more of the thiol groups of flour suspensions are susceptible to oxidation. It is pointed out, however, that the number of thiol groups/10^5 g. of flour, i.e. 6·0 moles, represents only 40% of the total determined in the presence of 8M-urea (Frater *et al.* 1960).

Table 4 shows the oxidation of the thiol groups of thiolated gelatin by iodate, bromate, persulphate and air. The rates of oxidation found are similar to those obtained with simple thiols (Hird & Yates, 1961; Frater *et al.* 1960), reduced gluten and flour. Oxidation by air is slower than that by the oxidizing agents used. Table 6 shows the appearance of disulphide bonds on oxidation of thiolated gelatin. As with reduced gluten there is formation of disulphide bonds. With increasing concentration of thiol groups there is an increase in the percentage yield of disulphide (Table 7). This is shown not only by the increased recovery of total sulphur as thiol plus disulphide but also by an increase in the moles of thiol oxidized/mole of iodate added. At concentrations of 5 and 10% of thiolated gelatin, gelling occurred.

Table 3. *Oxidation of reduced water-soluble proteins of flour*

Concentration of protein: 1 mg./ml. Oxidizing agents were used in a molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μmoles of thiol/100 mg. of protein.

Time (hr.)	Iodate	Bromate	Persulphate	Air
0	6·0	6·0	6·0	6·0
0·25	3·2	—	—	—
2	—	4·5	4·0	—
4	—	4·3	3·5	—
8	—	4·0	3·4	5·5

Table 4. *Oxidation of thiol groups of flour*

Concentration of flour: 1 g. in 20 ml. of water; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μmoles of thiol/10 g. of flour.

Time (hr.)	Iodate	Bromate	Persulphate
0	6·0	6·0	6·0
1	2·4	5·0	4·8
9	1·3	3·2	3·0

Table 5. *Oxidation of thiolated gelatin*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μmoles of thiol/100 mg. of protein.

Time	Iodate	Bromate	Persulphate	Air
0	15·5	15·0	15·5	16·6
5 min.	0	—	—	—
1 hr.	—	13·4	14·7	—
3 hr.	—	12·0	13·5	—
6 hr.	—	8·0	11·5	13·5

DISCUSSION

The results show that the thiol groups of the reduced proteins of flour and of thiolated gelatin are oxidized by iodate, bromate and persulphate. The rates of oxidation are similar to those obtained with low-molecular-weight thiols (Frater *et al.* 1960; Hird & Yates, 1961), i.e. very rapidly with iodate and slowly with bromate and persulphate.

Taking iodate as the example, the oxidation of a thiol may proceed between the two extremes (Hird & Yates, 1961).

$$6R\cdot SH + IO_3^- \rightarrow 3R\cdot S\text{–}S\cdot R + I^- + 3H_2O \quad (1)$$

$$R\cdot SH + IO_3^- \rightarrow R\cdot SO_3H + I^- \quad (2)$$

Thus the ratio of thiol oxidized to oxidizing agent at the equivalence point will vary between 6 and 1, and this ratio can therefore be a guide to the reactions occurring. At low concentrations of thiol groups the ratios, obtained with gluten and thiolated gelatin, are between 3·7 and 4·8, values similar to those obtained with cysteine (Hird & Yates, 1961). However, as the concentration of

Table 6. *Formation of disulphide bonds on oxidation of thiolated gelatin*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μmoles/100 mg. of protein.

	Iodate			Bromate			Persulphate		
Time	–SH	–S–S–	Total S (–SH + –S–S–)	–SH	–S–S–	Total S (–SH + –S–S–)	–SH	–S–S–	Total S (–SH + –S–S–
0	16·4	1·6	19·6	16·4	1·6	19·6	16·4	1·6	19·6
5 min.	0	6·5	13·0	—	—	—	—	—	—
24 hr.	—	—	—	6·5	5·5	17·5	0	6·5	13·0

Table 7. *Oxidation of thiolated gelatin*

Concentration of thiolated gelatin was as indicated. Quantities of iodate added were sufficient to oxidize approximately 50 and 75% of the thiol groups, assuming a molar ratio, thiol/oxidizing agent, 6/1. Ratios were calculated from μmoles of thiol groups oxidized to μmoles of iodate added. Results are expressed as μmoles/100 mg. of protein.

Concn. of thiolated gelatin (%)									
0·1					2·5				
SH oxidized (%)	–SH (μmoles)	–S–S– (μmoles)	Total S (μmoles)	Thiol/oxidizing agent	SH oxidized (%)	–SH (μmoles)	–S–S– (μmoles)	Total S (μmoles)	Thiol/oxidizing agent
Initial	16·6	1·3	19·2	—	Initial	16·3	1·6	19·5	—
45·8	9·0	3·8	16·6	4·5	37·5	10·2	3·4	17·0	5·0
72·4	4·6	6·2	17·0	4·8	54·0	7·5	4·5	16·5	4·9

Concn. of thiolated gelatin (%)									
5·0					10				
SH oxidized (%)	–SH (μmoles)	–S–S– (μmoles)	Total S (μmoles)	Thiol/oxidizing agent	SH oxidized (%)	–SH (μmoles)	–S–S– (μmoles)	Total S (μmoles)	Thiol/oxidizing agent
Initial	16·6	2·4	21·4	—	Initial	16·4	1·9	20·2	—
51·2	8·1	5·7	19·5	6·0	43·3	9·3	5·0	19·3	5·9
74·8	4·2	7·2	18·6	5·9	69·5	5·0	7·5	20·0	6·1

thiol groups is increased, the ratio goes up, corresponding to reaction (1). In the previous work with cysteine and in the present work with proteins the major product is found to be the disulphide but there is the likelihood of the corresponding sulphinic acid and sulphonic acid being formed, as happens with cysteine. The increase in disulphide formation with concentration suggests that the proximity of thiol groups undergoing oxidation will determine the products formed. This further suggests that it is difficult to extrapolate from the present experiments to a complex system such as dough, in which these oxidizing agents are used as improvers.

A further point arises from the type of disulphide formed. It has been shown by Benesch & Benesch (1958) that the oxidation by ferricyanide of thiolated gelatin at a concentration of 0·2% produces intramolecular disulphide bonds, and at a concentration of 5% the formation of intermolecular disulphide bonds causes gelling. In the present work iodate has also been shown to bring about gel formation at concentrations of thiolated gelatin of 5% and 10%. An increase in the number of intermolecular disulphide bonds resulting from oxidation of thiol groups of dough by improvers would, of course, increase its strength but the mere removal of thiol groups by *N*-ethylmaleimide and other thiol-blocking reagents is also sufficient to cause similar rheological changes in dough (Goldstein, 1957; Bloksma, 1958; Mecham, 1959; Bloksma & Hlynka, 1960; Frater *et al.* 1960; Mecham, Sokol & Pence, 1960; Frater, Hird & Moss, 1961). The changes in the strength of dough cannot therefore be wholly dependent on the formation of new intermolecular disulphide bonds, although these may make some contribution. It would seem, however, that the major effect of the removal of thiol groups by oxidizing agents is to be sought in the inhibition of exchange reactions involving such groups.

SUMMARY

1. The oxidation of the thiol groups of flour, reduced protein prepared from flour and thiolated gelatin has been studied.

2. Iodate, bromate and persulphate have been shown to oxidize thiol groups in the protein preparations. Iodate has been shown to oxidize at a faster rate than do bromate and persulphate.

3. When reduced gluten and thiolated gelatin were oxidized disulphide has been shown to be a product. When iodate oxidizes thiolated gelatin there is evidence that at lower concentrations of thiol groups oxidation products higher than disulphide are formed. At higher concentrations of thiol groups the product of oxidation is entirely the disulphide. Gelling occurs at these high concentrations.

4. The results are discussed in relation to the action of these oxidizing agents as improvers of the rheological properties of dough.

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A Direct Quantitative Paper Chromatography of Amino Acids and its Application to the Urinary Excretions of some Human Ethnic Groups

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The work of Dent & Harris (1951), Harris (1953), Harris & Warren (1953) and Datta & Harris (1953) has indicated the existence of characteristic patterns of urinary amino acid excretion in human and other mammalian species. As an item in the study of biochemical aspects of human racial differentiation it was planned to examine the excretions among physiologically normal members of ethnologically distinct human groups on Singapore Island and in the Federation of Malaya.

With so potentially extensive a programme it was proposed to employ direct transmission photometry ('minimum transmission' type) of ninhydrin-treated two-dimensional paper partition chromatograms for most of the assays. Established procedures were found to lack delicacy and accuracy, more in respect of colour development and photometry than of chromatography. A brief report of the preliminary work and details of the photometry refinements, which alone render the means of triplicate assays as reliable as those of some twelve replicates otherwise obtained, have been provided elsewhere (Lugg & McEvoy-Bowe, 1957, 1961; McEvoy-Bowe & Lugg, 1958, 1959). This article describes the chromatography, the closely controlled colour-development procedure (which, like the refined photometry, greatly reduces variability of results) and the preliminary treatment of urine samples, and records the results of the exploratory ethnic-group survey. Attention was directed principally to the excretion rates of glycine, taurine, β-amino*iso*butyric acid, alanine and glutamine, but those of cystine (determined polarographically) have also been included.

EXPERIMENTAL

Chromatography. Square sheets (20 cm. × 20 cm.) of Whatman no. 1 filter paper were used in two-dimensional ascending-front separations. Perforated at the corners, they were fitted usually 12 at a time with glass spacing sleeves on the rails of a stainless-steel frame resting in a trough of this alloy (cf. Datta, Dent & Harris, 1950), at the bottom of a glass tank (36·5 cm. × 26·5 cm. base and 24·5 cm. height; as supplied by Shandon Scientific Co. Ltd., London, W. 10) fitted with a glass sealing lid. Aluminium-alloy frames and troughs (as used by Datta *et al.* 1950) were found unsatisfactory. Climatic conditions made it necessary to control, thermostatically, the temperature of the tank enclosure (at 29·5±0·5°).

The initial spot was a 5·0 mm. disk with centre 4·5 cm. diagonally inward from a corner, applied as evenly as possible from an Agla (Burroughs Wellcome and Co. Ltd., London, N.W. 1) micrometer syringe, and dried with warm air from a hair-drier. The spot was briefly exposed to ammonia gas and excess blown away.

So-called 'single-phase' solvent systems, claimed in the literature to be advantageous, were used for each 'run'. They appear to function as conventional double-phase systems in association with sheet fibres (Martin, 1949). For the first run, which was stopped with the front approx. 1 cm. from the top, the solvent was prepared from water-saturated phenol by dissolving in it 10% (w/v) phenol, 0·04% (w/v) 8-hydroxyquinoline as heavy-metal chelating agent (Block, 1950), and 0·5% (w/v) 2:6-lutidine. With this solvent the sheets were used as supplied, as prior washing conferred no benefit. For the second run, stopped with the front almost at the top, the solvent (Block, Durrum & Zweig, 1955) was prepared by mixing 55 vol. of redistilled 2:6-lutidine with 25 vol. of 95% ethanol and 20 vol. of water. Diethylamine (1·5 ml.) was placed in the bottom of the tank, as direct addition to the solvent appreciably increased the 'background'.

Solvents were removed by blowing air at room temperature (26–30°) over the sheets until characteristic odours were dispelled (approx. 12 hr. for the first and 1 hr. for the second solvent).

Reversing the order of solvents improved band-shape regularity but capriciously affected the tints of the final colour bands. Flow rates and band-shape regularity varied with batches of paper (supplied by H. Reeve Angel and Co. Ltd., London, E.C. 4). Batches of paper requiring approx. 7 hr. for the first run were selected.

Use of the ninhydrin reagent. The acidity or basicity of materials on the paper affects the absorption spectra of the final bands (Thompson, Zacharius & Steward, 1951; Lewis, 1952). If solubilities of band substances in the reagent are not very low they may flow on immersion of sheets and even be dissolved off.

Stock solutions of ninhydrin (3%, w/v, in 95% ethanol) were kept at 3° in the dark for not more than a few weeks. The final reagent was prepared immediately before use by mixing 5 vol. of stock solution with 5 vol. of 95% ethanol, 39 vol. of redistilled chloroform and 1 vol of 2:6-lutidine. Sheets were immersed for 2 sec., the bulk of solvents evaporating during withdrawal. They retained approx. 0·05 mg. of ninhydrin/cm.^{2} of sheet, a quantity well in excess of requirements. They were then exposed, on a frame, to a vigorous current of air at room temperature for 1–2 min. before heat treatment (see below).

Colour development. The humidity of the reaction space, the temperature, and the time allowed, are all of great importance, as suggested by the work of Redfield & Barron (1952) and of Wellington (1953). Many experiments have been carried out to determine optimum conditions for assay and only the crucial ones are recorded (see Results). The optimum conditions were found to be as follows.

Room air was blown at 21 l./min. by a rotary blower [supplied by Baird and Tatlock (London) Ltd.] through 1 l. of acidulated water maintained at a definite temperature (43° recommended) in a 2 l. flask and thence, via a steam-jacketed condenser tube, to the bottom of a thermostatically regulated oven (90° recommended), vented at the top, and of internal dimensions 40·5 cm. × 30·5 cm. base and 43 cm. height, accommodating the chromatograph frame fitted with only three assay and three alternating standard sheets to facilitate air flow. The recommended period of treatment is 30 min. In the experiments leading to these recommendations steam was admitted to the jacket sufficient to raise the entering air to within a few degrees of that of the oven, and the lowest relative humidity, virtually zero except for sorbed moisture on the sheets, was provided by substituting for the 2 l. flask a column of anhydrous calcium chloride pellets.

Photometry. Substance bands were identified primarily by their R_F values as compared with those of known substances on standard sheets run simultaneously, and secondarily by the tints, which were somewhat characteristic. Developed sheets and excised portions thereof, or both, were kept in the dark (and for no more than several hours) before photometry.

Transmission photometry of the 'minimum transmission' type was employed, the densitometer (McEvoy-Bowe & Lugg, 1958) permitting precise positional replacement of the excised sheet portions after bleaching, and thus correcting for textural irregularities in determinations of band and background transmittances. Bleaching was carried out by exposing the excised portions to chlorine gas from moist bleaching powder until they appeared the same as unused sheet similarly exposed, the optical properties of unused sheet actually being unaffected by this treatment. Galvanometer deflexions were sensibly proportional to the intensity of monochromatic light impinging on the selenium barrier-layer photocell. If R is the deflexion for sheet with band and background, B that for sheet alone there, R' for the sheet with background and B' for sheet alone there, then, if all light reflected back is restored to the sheet, R/B is the transmittance of band and background and R'/B' is the background transmittance, and to close approximation $RB'/R'B$ is the transmittance (T) of band alone where measured (Lugg & McEvoy-Bowe, 1961).

With the simple optical system actually employed in this work (with near-white light, with no restoration of reflected-back light) the above relationships do not strictly obtain, but the terms may be computed as if they do and a simple function of the computed band transmittance, T, is found to be related linearly to the quantity, Q, of a substance applied in the initial spot (see Results and Discussion).

Collection, preparation and assay of urine samples. A 24 hr. collection of urine with toluene as preservative was obtained from each subject. Storage (at 4°) was kept as short as possible to minimize decomposition, especially of glutamine (see Stein, 1953). Portions were taken for the estimation of cystine (polarographic method of Reed, 1942), and for desalting (to avoid distortion of bands) before chromatography.

Clarkson & Kench (1956) found desalting with ion-exchange resins satisfactory for many amino acids. Losses of taurine and of glutamine (by deamidation) may be substantial and variable, but complete recovery of glutamine has been claimed for the modification of Thompson, Morris & Gering (1959). For assay of taurine, β-amino*iso*butyric acid, alanine and glutamine, electrolytic desalting by the method of Astrup, Stage & Olsen (1951) was adopted, but the cathode compartment of the desalter was fitted with a water jacket. A 10 ml. urine portion mixed with 10 ml. of distilled water was electrodialysed with a starting current of usually 1·0–1·2 A. After the current had fallen to 0·6 A (approx. 30 min.) the process was continued for a further 15 min., the current having then fallen to 0·25–0·5 A. The fluid was withdrawn and, with washings, diluted to 25 ml., and of this volume a suitable portion was applied as initial spot to a sheet. The portion was usually in the range 0·0005–0·001 (represented by 10–20 μl.) and depended on the volume of 24 hr. urine and the size of the subject. As hippuric acid is partially hydrolysed to glycine during the electrolytic desalting process (see Results), ion-exchange desalting by the method of Clarkson & Kench (1956) was employed in estimating the glycine contents of urines.

Subjects. Ten subjects were taken at random from somewhat restricted categories of healthy male members in the 17–50 year age range of each of the European (university teaching staff), Southern Chinese (resident university students) and Sēnoi (transferred several years before from a jungle to a reserve environment) ethnic groups living on Singapore Island or in the Federation of Malaya. The European sample included one Maltese. The Sēnoi sample included members of the Sēmai and Temiar divisions (see Williams-Hunt, 1952).

RESULTS

The function of T to which Q was found to bear a linear relationship is $(1/T)-1 = a$. However, a may also be taken as a measure of band colour intensity under various conditions of colour development, and $(B'/R')-1$ may be taken as the corresponding measure of background colour intensity.

Variation of colour intensity with conditions of colour development. In testing the effects upon colour development of varying the heating period, the relative humidity and the temperature, mixtures of the five substances were taken for chromatography in such quantities as would not confuse a graphical presentation of results. The effects of varying the heating period at 90° and 12·3% relative humidity, are shown in Fig. 1. The effects of varying the relative humidity at 90° and for a 30 min. heating period, are shown in Fig. 2, but values of a at 'zero' relative humidity are undoubtedly too high because of the sorbed moisture introduced with the sheets.

The general effects of reducing the temperature, from 90° to 80° to 70°, upon the conditions for optimum colour development, were to increase the period required at constant relative humidity and, with reasonably lengthy constant period, to increase the relative humidity required.

Relationships between Q and T; variability of results. It has been shown (Lugg & McEvoy-Bowe, 1961) that even if the parent substances behave ideally in the chromatography, and sheet and colour substances on it absorb 'entering' light (namely incident light less that reflected back and lost) in a manner conforming with Lambert's and Beer's laws, the computed absorbance ($D = -\log T$) of the band when incident light-intensity is constant can be proportional to Q only if the reflected light is restored. The proof presumes that colour substance density is proportional to parent substance density (see Discussion). Standard chromatograms were run together in triplicate. Seven such experiments were made at the 2 and 20 μm-mole and five at the 4 and 10 μm-mole quantities. Overall mean D values at each quantity were examined, and for all five substances were found to increase with Q but disproportionately slowly. This is to be expected with the optical system used if the above-mentioned circumstances actually obtain.

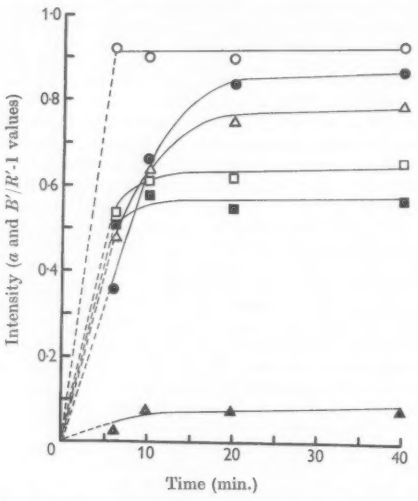

Fig. 1. Relationships between band colour intensities (as a values) for five substances chromatographed at unspecified but constant quantities and of 'background' colour intensities (as $B'/R'-1$ values) and time, the ninhydrin reaction being conducted at 90° in air maintained at 12·3% relative humidity. ○, Glycine; △, taurine; ●, β-amino*iso*butyric acid; □, alanine; ■, glutamine; ▲, 'background'.

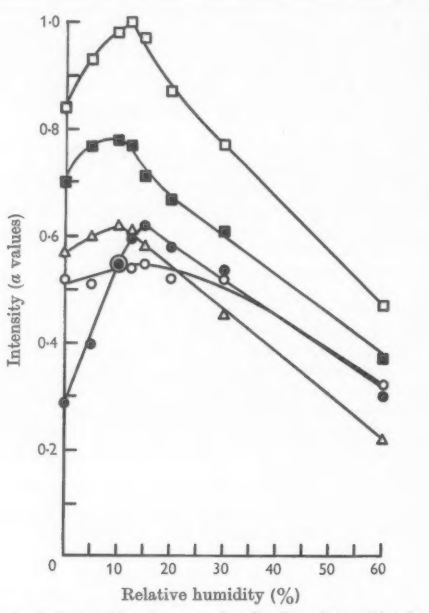

Fig. 2. Relationships between band colour intensities (as a values) for five substances (chromatographed at unspecified but constant quantities) and relative humidity, the ninhydrin reaction being conducted in air at 90° for a period of 30 min. ○, Glycine; △, taurine; ●, β-amino*iso*-butyric acid; □, alanine; ■, glutamine.

An exhaustive statistical analysis of the original data for β-amino*iso*butyric acid at the 2 and 20 μm-mole quantities is given elsewhere (Lugg & McEvoy-Bowe, 1961) together with a discussion of the physical significance of the linear relationship between Q and a. In Table 1 is a digest of the original data which established the semiempirical equation:

$$Q = k'''a = k'''[(1/T - 1)], \quad (1)$$

where k''' is a constant for the substance concerned, in the range 2–20 μm-moles under the prevailing conditions.

This equation is considered further in the Discussion together with the variabilities of a. Therein is stressed the advisability of making 'within-experiment' assays, to which recourse was had [by using equation (2), derived from equation (1)] in the urine analyses:

$$Q_{(assay)} = Q_{(std.)}\, a_{(assay)}/a_{(std.)}, \quad (2)$$

where $a_{(assay)}$ and $a_{(std.)}$ are closely similar means from triplicate chromatograms run together, the % standard error (S.E.) of $Q_{(assay)}$ being $\sqrt{2}$ times the appropriate value obtained by interpolation from the final column of Table 1.

Effects of desalting upon recoveries. Experiments were made to test recovery (a) from four simulated urines containing (in addition to these five substances), urea, creatinine, uric acid, Na^+, K^+, NH_4^+, Ca^{2+}, Cl^-, SO_4^{2-}, $H_2PO_4^-$ and HPO_2^{2-} ions, all at normally occurring concentrations, and (b) from ten actual urines in which additional quantities of the five substances had been dissolved.

To within the standard errors of the means the recoveries in the (a) series were complete, except for taurine with a mean recovery of 92 % (S.E. 1 %) which agrees closely with the 93 % recovery found by Astrup *et al.* (1951) after desalting in a 0·15M-sodium chloride medium. In the (b) series the mean recoveries of glycine, alanine and glutamine were complete, but the mean recovery of β-amino-*iso*butyric acid was only 80 % (S.E. 2 %) and that of taurine appeared excessive, 107 % (S.E. 5 %).

Work on the Sēnoi and some European urine samples was completed early in the survey, the urines being desalted only electrolytically. The mean 24 hr. excretion of glycine by the European subjects, 3·43 m-moles, was about twice that reported by Stein (1953), who used ion-exchange chromatographic analysis. This and other evidence suggested that urinary glycine conjugates, such as hippuric and glycocholic acids, might undergo some hydrolysis in the desalter and lead to spuriously high values.

In pure solution hippuric acid was not affected in the electrolytic desalter, but in the presence of sodium chloride there was appreciable liberation of glycine, the effect increasing with the initial salt concentration and with time in the desalter. As fresh collections from the Sēnoi subjects could not be obtained their glycine values were discarded, but in repeated work with European subjects and

Table 1. *Relationship between quantity (Q) of applied substance and the measure (a) of band colour intensity, with information derived from analyses of variance*

Triplicate chromatograms of all five substances were run together in each experiment: seven experiments at the 2 and 20 μm-mole and five experiments at the 4 and 10 μm-mole quantities. c-v, Coefficient of variation.

Substance	Q (μm-moles)	Mean a	Within-expt. c-v (%)	Total c-v (%)	S.E.M. of triplicate within expt. (%)
Glycine	2	0·1147	16	17	9·2
	4	0·2146	10	11	5·8
	10	0·5425	8·4	12	4·8
	20	1·0868	5·7	8·1	3·3
Taurine	2	0·1187	8·4	12	4·9
	4	0·2412	4·5	15	2·6
	10	0·6014	7·0	14	4·0
	20	1·2477	5·6	11	3·2
β-Amino*iso*butyric acid	2	0·1171	13	13	7·5
	4	0·2052	7·4	9·6	4·3
	10	0·5591	6·2	10	3·6
	20	1·1556	5·0	5·3	2·9
Alanine	2	0·2128	10	11	5·8
	4	0·3829	8·5	9·4	4·9
	10	0·9327	5·2	9·0	3·0
	20	1·8861	5·0	7·8	2·9
Glutamine	2	0·1476	9·2	12	5·3
	4	0·2556	8·9	9·1	5·1
	10	0·6688	5·6	13	3·2
	20	1·3297	5·6	5·9	3·2

Table 2. *Rates of excretion of substances by individual members of the three ethnic groups, Chinese* (C), *European* (E) *and Sēnoi* (S), *with means and estimates of group standard deviations* (S.D.)

		Excretion rates (m-moles/24 hr.)					
Subject	Body wt. (kg.)	Glycine	Taurine	β-Amino*iso*-butyric acid	Alanine	Glutamine	Cystine
C1	55·4	1·52	0·90	0·30	0·60	0·67	0·17
C2	54·4	1·49	1·03	0·36	0·49	0·67	0·12
C3	58·1	0·91	0·91	0·14	0·37	0·46	0·09
C4	61·7	2·31	1·37	0·82	0·68	0·71	0·08
C5	81·7	0·91	2·45	0·36	0·34	0·70	0·19
C6	56·3	1·06	1·24	0·10	0·37	0·69	0·17
C7	50·8	2·02	1·25	0·10	0·24	0·49	0·06
C8	54·4	1·23	1·19	1·54	0·34	0·52	0·06
C9	56·7	1·66	1·80	0·48	0·41	0·57	0·14
C10	60·8	1·12	0·94	0·38	0·47	0·22	0·09
Mean	59·03	1·423	1·308	0·458	0·431	0·570	0·117
S.D.	8·575	0·4715	0·4804	0·4359	0·1318	0·1543	0·0481
E1	83·9	1·05	1·02	0·25	0·30	0·68	0·26
E2	63·5	1·40	0·22	0·30	0·17	0·54	0·20
E3	69·0	1·70	0·65	0·19	0·37	0·81	0·18
E4	73·5	1·44	0·69	0·18	0·32	0·61	0·22
E5	60·3	2·96	0·93	0·15	0·34	0·97	0·24
E6	62·6	1·04	0·76	0·18	0·28	0·32	0·24
E7	71·2	1·26	1·17	0·03	0·36	0·52	0·21
E8	70·8	2·20	1·88	0·27	0·49	0·84	0·33
E9	64·0	3·16	0·92	0·05	0·29	0·55	0·15
E10	74·4	1·43	1·64	0·11	0·50	0·93	0·25
Mean	69·32	1·764	0·988	0·171	0·342	0·677	0·228
S.D.	7·074	0·7609	0·4839	0·0894	0·0980	0·2070	0·0492
S1	49·5	—	0·61	0·79	0·08	0·12	0·13
S2	49·9	—	1·14	0·36	0·16	0·25	0·33
S3	43·6	—	1·06	1·02	0·11	0·18	0·12
S4	62·6	—	1·42	1·54	0·28	0·35	0·20
S5	49·5	—	0·74	0·42	0·09	0·19	0·15
S6	61·7	—	0·97	0·40	0·17	0·19	0·15
S7	55·4	—	0·29	0·04	0·05	0·10	0·07
S8	50·8	—	0·55	0·25	0·19	0·19	0·14
S9	52·2	—	1·04	1·25	0·11	0·25	0·15
S10	51·3	—	0·38	1·96	0·13	0·22	0·18
Mean	52·65	—	0·820	0·803	0·137	0·204	0·162
S.D.	5·804	—	0·3634	0·6256	0·0662	0·0706	0·0684

in the work with Chinese subjects, glycine was estimated only after ion-exchange desalting (as previously described).

Ethnic-group data. Table 2 records the rates of excretion of the six substances by members of the three ethnic groups. β-Amino*iso*butyric acid values have been corrected for 20% loss (see Discussion).

DISCUSSION

However closely the ninhydrin reaction may come to yielding diketohydrindylindene–diketohydrindamine stoicheiometrically in suitably constituted solutions (Troll & Cannan, 1953; Moore & Stein, 1954), the requisite conditions cannot be provided satisfactorily on paper sheets. The reaction is customarily carried out by heating the ninhydrin-impregnated sheets at a definite temperature for a definite period with usually, but not always, some deliberate control of humidity. Even among the α-amino-α-carboxylic acids the absorption spectra of the colour substances differ slightly and the molecular extinction indices at any chosen light-frequency may vary appreciably. However, whatever the reaction(s) may be, there is evidence (Moore & Stein, 1948) that the quantities of colour substance(s) produced are proportional to the quantities of parent substance reacting with the excess of ninhydrin.

Results of the experiments reported here are seriously in conflict with those of Redfield & Barron (1952) that led them to conclude that the optimum relative humidity is $50 \pm 10\%$ at 90°, but they may be in harmony with those of Wellington (1953) which indicated an optimum relative humidity of $40 \pm 3\%$ at $20 \pm 1°$. It is apparent from Fig. 2 and cognate data that at any given temperature both the magnitude and degree of control of the relative humidity for satisfactory colour development are critical.

The electrolytic desalting loss of β-amino*iso*butyric acid which occurred in the (*b*), but not the (*a*), series of experiments has been attributed to reaction with one or more substances present in normal but not in simulated urines, and the correction for 20% loss in urine assays was based upon this interpretation. The presence of taurocholic acid in urines might lead to spuriously high taurine assays after electrolytic desalting, but this possibility cannot explain the apparent 7% 'gain' of taurine in the (*b*) as compared with the 8% loss in the (*a*) series of experiments: in the circumstances no correction was applied to the urine assay values.

Standard curves for assay purposes may be constructed from the Q and $-\log T\ (=D)$ values. Linear regression equations based upon the Q and the $1/T$ or the a values may similarly be used, though there are certain theoretical objections to deriving and using them when either variate (here Q) has values confined within controlled experimental limits. Both approaches involve acceptance of the usually substantial 'between-experiment' contributions to total variance (see Table 1). An alternative approach (adopted in the present work), which excludes these contributions, is to accept equation (1) as adequately expressing the relationship between Q and a and to employ it, as equation (2), in 'within-experiment' estimations. Incidentally, with chromatograms of sugars on the same type of paper and similar densitometry of the colour bands produced by aniline hydrogen phthalate treatment, equation (1) is equally valid (E. McEvoy-Bowe, unpublished work, 1957).

As was indicated earlier, failure to employ the refined photometry used in this work would require some 12 'within-experiment' replicates to furnish means with percentage standard errors as low as those shown in Table 1. Yet these errors (range 2·6–9·2% with 4·5% mean) are only about one-third of those reported by Block *et al.* (1955) for the mean of 12 replicates, namely 10–15%. This further gain in reliability is attributed partly to better control of the chromatography but mostly to rigorous control of colour development. All factors combined make the mean of triplicates as reliable as the mean of about 100 replicates in the procedure recommended by Block *et al.* (1955).

The purpose of the survey was to disclose likely differences in excretion patterns for more extensive future studies. As in other work with larger samples from these three groups (Lugg & Bowness, 1957) the European subjects were usually the heaviest and the Sēnoi the lightest. More subtle differences than those suggested by Table 2 might be revealed by relating the excretions to (for example) creatinine excretions and by working with larger samples.

Individual values, means and standard deviations, considered as they stand and also with some reference to body weight, suggest that significance might be attached (i) to the low cystine excretions in the Chinese sample and to the low alanine and glutamine excretions in the Sēnoi; (ii) to the incidence of notably higher glycine:taurine ratios in the European than in the Chinese sample, and (iii) to several aspects of the differences in distributions of β-amino*iso*butyric acid values.

In respect of (i) the cystine values would include any other disulphides and any thiol compounds (unlikely to be present), or both, in the urines. The European values correspond closely with those found by Reed (1942) and, like them, are considerably greater than those found by Stein (1953) employing an ion-exchange chromatographic method. The chemical significance of this discrepancy is uncertain (Evered, 1956). The statistical significance of the difference between the European and Chinese means is very high (Student's $t = 4{\cdot}95, P = < 0{\cdot}001$), nor would it be diminished vastly by referring excretion rates to a weight basis. There is no instance of 'cystinuria', classical or otherwise (see Dent & Harris, 1951), in any of the samples. The generally low alanine and glutamine values in the Sēnoi sample may be merely a reflexion of the relatively protein-poor (though not inadequate) diet of the group.

No indication of (ii) has emerged from previous studies. High glycine excretion rates appear to be frequent among Europeans (Dent & Harris, 1951). But, whereas Evered (1956) was inclined to associate elevated taurine excretion rates with protein-rich diets, it is noteworthy that (especially in relation to body weight) the mean rate in the Sēnoi sample is not low, and in the Chinese sample relatively high, compared with that in the European. The evidence, viewed as a whole, strongly suggests the involvement of endogenous, racial factors in the excretion of taurine (such factors operate in populations of mice; Harris & Searle, 1952) as well as of glycine.

With reference to (iii), although there is evidence (Fink, Henderson & Fink, 1951) that the excretion of β-amino*iso*butyric acid by the rat is subject to the effects of endogenous (dietary) factors, these are believed to play a negligible role in normal human subjects maintained on normal diets. Within-sample distributions of values recorded in Table 2 are shown in Fig. 3. In constructing the frequency polygons the disturbing effects of chance in sampling with such small samples have been reduced by distributing the observations (as fractions mostly) between single-valued classes (Lugg & Bowness, 1957) instead of conventionally placing each wholly within a class span.

A bimodal distribution of human subjects into 'low' and 'higher' excretors of β-amino*iso*butyric

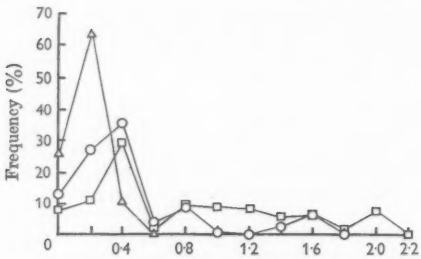

Fig. 3. Frequency distributions of β-aminoisobutyric acid excretion rates in the three ethnic groups. ○, Chinese; △, European; □, Sēnoi.

acid was suggested by the original work of Crumpler Dent, Harris & Westall (1951) and supported by that of Harris (1953). If, from the appearance of Fig. 3, the 'low' excretors are accepted as those excreting less than 0·6 m-mole/24 hr. then, reverting to the original data, the European sample contained no 'higher' excretors, whereas the Chinese contained two (C4 and C8) and the Sēnoi five (S1, S3, S4, S9 and S10). The statistical significance of the differences in incidence of 'low' and 'higher' excretors is appreciable: χ^2 (2 degrees of freedom) = 7·07, $P = 0{\cdot}03$. Probabilities of encountered or greater differences (in either direction) in the pair comparisons, computed by the 'exact' method (see Fisher, 1954), are: $P = 0{\cdot}47$, for Chinese with European; $P = 0{\cdot}35$, for Chinese with Sēnoi; and $P = 0{\cdot}032$, for European with Sēnoi.

Only in the European–Sēnoi comparison is the statistical significance fairly high. Apart, however, from this type of differentiation, the European 'low' excretors (especially in relation to body weight) appear to be differentiated somewhat from the 'low' excretors in the other two ethnic groups.

Estimates, by varying criteria, of the incidence of higher excretors among populations of basically Caucasoid origin have varied: 4·8% (Crumpler *et al.* 1951), 8% (Fink *et al.* 1951), 9·6% (Harris, 1953) and 10·2% (Allison, Blumberg & Gartler, 1959). The incidence is clearly low, and the lack of a 'higher' excretor in the present small European sample is not contrary to expectations.

Estimates by Allison *et al.* (1959) of the incidence of 'higher' excretors among populations generally recognized as of basically Mongoloid origin are, 56 and 59% for two 'Amerind' groups and 23% for Alaskan Eskimos. Sutton & Clark's (1955) findings from their comparative study of excretion rates of β-aminoisobutyric acid and other substances in European and Chinese groups are open to question because of the divergence of their assay values from those of other investigators.

Closer examination of Fig. 3 suggests that the 'higher' excretors themselves may not form a unimodally disperse fraction, a possibility already implicit in the results of Fink *et al.* (1951). The Chinese sample might well be regarded as containing one 'high' and one 'very high' excretor, and the Sēnoi as containing two or three 'high' and (correspondingly) three or two 'very high' excretors, depending upon where the division might be made.

All in all, the β-aminoisobutyric acid data, if not the rest, are in conformity with earlier evidence (for brief review see Lugg, 1957) that the Sēnoi are, like the Southern Chinese, of basically Mongoloid origin.

Departures from unimodality of distributions of β-aminoisobutyric acid excretion rates in human subjects have been attributed (Crumpler *et al.* 1951; Harris, 1953) to inherited variability in the renal threshold levels. The evidence supporting this interpretation is substantial; but the actual pattern of inheritance would now seem to be far more complex than has formerly been supposed.

SUMMARY

1. Conditions of running two-dimensional paper-sheet partition chromatograms of mixtures of amino acids (and derivatives), and of developing band colorations with ninhydrin, have been studied with a view to furnishing sheets suitable for direct transmission photometry with a refined type of densitometer.

2. The recommended conditions, coupled with the simplest form of photometry afforded by the densitometer, make the mean of triplicate assays as reliable as the mean of about one hundred replicates in the procedure recommended by Block *et al.* (1955).

3. By the refined methods an exploratory survey has been made of the rates of urinary excretion of glycine, taurine, β-aminoisobutyric acid, alanine and glutamine by members of one aboriginal (Sēnoi) and two civilized (European and Southern Chinese) ethnic groups living on Singapore Island and in the Federation of Malaya. The work involved a preliminary study of 'desalting' techniques. Cystine excretion rates, estimated polarographically, were included in the survey.

4. Attention has been drawn to the more salient aspects of ethnic-group differentiation and correspondence suggested by the results of the survey.

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Flight-Muscle Mitochondria of *Lucilia cuprina* and *Musca domestica*

ESTIMATION OF THE PYRIDINE NUCLEOTIDE CONTENT AND OF THE RESPONSE OF RESPIRATION TO ADENOSINE DIPHOSPHATE

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The ability of many species of flying insects to increase their consumption of oxygen during flight and the factors controlling respiration during rest and activity have been the subject of much research (for a recent summary, see Sacktor, 1958; Bucher, Klingenberg & Zebe, 1958). The production of adenosine triphosphate by oxidative phosphorylation in the flight-muscle mitochondria is a most important aspect of energy transformation in such animals (Sacktor, 1954; Slater & Lewis, 1954), as in many other organisms. However, Sacktor and co-workers have described certain unusual features of both the control of respiration with α-glycerophosphate, which is oxidized most readily by mitochondria from insect muscles (e.g. Chance & Sacktor, 1958), and in the composition of the electron-transport chain of particles isolated from thoraces of the fly *Musca domestica* L. With preparations from mature flies (3–7 days old) they found that the oxidation of α-glycerophosphate was independent of a supply of adenosine diphosphate (Sacktor, 1958; Sacktor & Cochran, 1958; Estabrook & Sacktor, 1958) and that pyridine nucleotide coenzymes were not detectable by the

spectrophotometric method used (Chance & Sacktor, 1958; see also Chance & Williams, 1955). Bucher *et al.* (1958) have queried the physiological significance of these findings; their preparations of mitochondria from the locust (*Locusta migratoria*) oxidized α-glycerophosphate more rapidly when adenosine diphosphate was present and contained a relatively high concentration of diphosphopyridine nucleotide (Klingenberg & Bucher, 1959), as did mitochondria isolated from the blowfly *Calliphora* (Price & Lewis, 1959).

This paper describes an investigation of both the concentration and reactivity of the pyridine nucleotides of flight-muscle mitochondria of *Musca* and the conditions in which the oxidation of α-glycerophosphate is controlled by adenosine diphosphate.

MATERIALS AND METHODS

Diphosphopyridine nucleotides, ADP, ATP (as sodium salts) and hexokinase (160 000 K.M. units/g.; Kunitz & McDonald, 1946) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents used were of analytical grade.

Flies. Breeding of *Lucilia cuprina* Wied. was carried out by the method of L. Barton-Browne (unpublished work); adults were allowed to feed freely on sucrose and water and were kept at 25°. They were collected for preparation of mitochondria after inactivation at 5° for 5 min. Adults of *Musca domestica* L. were obtained from a single batch of pupae and treated in the same fashion.

Preparation of mitochondria. This was performed at 0–5°. Heads and abdomens were removed from the flies (mixed sexes) and thoraces were collected in a mortar containing an ice-cold suspension medium of 0·3 M-sucrose and 2 mM-sodium ethylenediaminetetra-acetate (EDTA). The tissue was crushed gently for about 2 min. with a pestle, the brei squeezed through four layers of muslin and the resulting suspension centrifuged for 3 min. at 150*g*. Mitochondria were sedimented from the supernatant fluid at 5000*g* for 10 min. Particles used in the experiments on respiratory control were washed twice, by resuspending in and centrifuging from the suspension medium, then made to the required volume with this medium; those required for pyridine nucleotide analyses were washed similarly in a saline medium (KCl, 0·173 M; EDTA, 2 mM). In one experiment, nicotinamide (50 mM) was added to this medium (see text). Microscopic examination of these preparations showed little contamination by either cytoplasmic debris or nuclei.

Measurement of respiration. The conventional Warburg technique was used with air as the gas phase. The final incubation medium (total volume, 2·5 ml., pH 7·2) contained 5 mM-$MgCl_2$, 5 mM-potassium phosphate buffer (pH 7·2), 1 mM-EDTA, 0·3 M-sucrose and the appropriate concentration of mitochondria; ADP (10 mM) or KCl (20 mM), together with the substrates (at pH 7·2), were added from the side arm after 10 min. preincubation at 25° unless otherwise noted in the Tables. In all the experiments the centre well contained 0·15 ml. of NaOH (2 N) and filter paper.

q_{O_2} values are expressed as μl. of O_2 consumed/mg. of protein/hr. The term 'respiratory control' has been used to imply that maximum rates of O_2 uptake with particular substrates require the presence of ADP. (For discussion of the use of this term, see Chance & Baltscheffsky, 1958.) The 'respiratory control ratio' is the quotient:

rate of O_2 uptake with ADP/rate of O_2 uptake without ADP.

Estimation of pyridine nucleotides. These were estimated by the fluorimetric method of Bassham, Birt, Hems & Loening (1959); with all preparations it was necessary to use the enzymic method for producing the 'blanks' (see Bassham *et al.* 1959). Pyridine nucleotide retention by the mitochondria in an incubated suspension was determined by the method of Birt & Bartley (1960*a*).

The following abbreviations have been used: DPN^+, DPNH and DPN, oxidized, reduced and total diphosphopyridine nucleotides respectively; TPN^+, TPNH and TPN, oxidized, reduced and total triphosphopyridine nucleotides respectively; PN^+, PNH and PN, DPN^+ plus TPN^+, DPNH plus TPNH, DPN plus TPN respectively.

Estimation of protein. Protein was estimated by the method of Cleland & Slater (1953).

RESULTS

Pyridine nucleotide content of mitochondria isolated from the thoraces of Lucilia cuprina *and* Musca domestica. The amounts of the coenzymes found in isolated flight-muscle mitochondria are given in Table 1. The detection of TPN^+ and TPNH was difficult, but as each experiment indicated the presence of at least one form of this nucleotide, the existence of very low concentrations of the coenzyme in these mitochondria was established with reasonable certainty.

Nicotinamide increased the total recovery of the coenzymes slightly, by preserving DPN^+ (Table 1). Because of the complications introduced by its addition in metabolic experiments (Birt & Bartley, 1960*a*), it was omitted from routine preparations.

Table 1. *Pyridine nucleotide content of fly-muscle mitochondria*

(*a*) Mitochondria from *Musca domestica*; values are the means of six estimations with different preparations. The range of values is given in parentheses. (*b*) Single estimation of *Musca* mitochondria prepared in a medium containing nicotinamide (50 mM). (*c*) Mitochondria from *Lucilia cuprina*; mean and range of three estimations. Flies used in all these experiments were 4–18 days old.

	Coenzyme content (μm-moles/mg. of protein)		
	(*a*)	(*b*)	(*c*)
DPN^+	2·15 (1·7–2·4)	2·52	3·0 (1·5–3·8)
DPNH	0·20 (0–0·4)	0·15	0·05 (0–0·17)
DPN	2·35	2·67	3·05
TPN^+	0·03 (0–0·05)	0·01	0·11 (0·07–0·20)
TPNH	0·13 (0–0·20)	0·10	0·10 (0–0·20)
TPN	0·16	0·11	0·21

Table 2. *Effect of added substrates on the pyridine nucleotide content and respiration of* Musca *and* Lucilia *mitochondria*

Expts. 1–3 were with *Musca* mitochondria; Expts. 4 and 5 were with *Lucilia* mitochondria. Concn. of all substrates, except pyruvate (10 mM): 50 mM. Suspensions were incubated for 10 min. at 25° in the Warburg apparatus with the substrates shown. Flies used were 3–20 days old.

Expt. no.	Conditions of incubation	Coenzyme content (μm-moles/mg. of protein) DPN⁺ + TPN⁺	DPNH + TPNH	DPN + TPN	$\frac{DPNH+TPNH}{DPN^+ + TPN^+}$	q_{O_2}*
1	No substrate; 0°; 0 min.	1·85	0	1·85	0	—
	Succinate	0·64	0·78	1·42	1·2	27
	α-Glycerophosphate	1·4	0	1·4	0	53
	Pyruvate plus malate	1·3	0·28	1·58	0·2	23
2	No substrate; 0°; 0 min.	1·40	0·04	1·44	0·0	—
	Succinate	0·90	0·34	1·24	0·37	73
	Succinate plus malate	0·95	0·20	1·15	0·21	7
	Glutamate plus malate	1·50	0	1·54	0	7
3	No substrate; 0°; 0 min.	2·40	0·7	3·17	0·3	—
	Succinate	1·38	0·8	2·18	0·6	34
	α-Glycerophosphate	0·90	0·97	1·87	1·1	50
4	No substrate; 0°; 0 min.	2·50	0·15	2·65	0·6	—
	Succinate	0·95	1·25	2·15	1·3	69
	α-Glycerophosphate	1·80	0·38	2·18	0·2	101
	Pyruvate plus malate	1·0	1·15	2·15	1·2	40
5	No substrate; 0°; 0 min.	2·85	0·20	3·05	0·07	—
	Succinate	1·90	1·10	3·0	0·6	30
	Succinate plus ATP	1·75	1·15	2·9	0·7	31
	α-Glycerophosphate	2·00	1·00	3·0	0·5	91
	α-Glycerophosphate plus ATP	1·85	1·05	2·9	0·6	93

* q_{O_2} values for oxidation of α-glycerophosphate are all low as the flasks contained 10–15 mg. of protein in a final volume of 2·5 ml.; the O_2 uptakes quoted for this series of experiments were measured with particles incubated in the saline medium (see text).

Table 3. *Retention of pyridine nucleotides by mitochondria during incubation*

For experimental details see Materials and Methods section.

Nucleotide estimated in	Coenzyme content (μm-mole/mg. of protein) 0°; 0 min. PN⁺	PNH	PN	No substrate; 25°; 15 min. PN⁺	PNH	PN	Succinate; 25°; 15 min. PN⁺	PNH	PN
(*a*) Whole suspension	1·9	0·05	1·95	1·65	0	1·65	0·65	0·85	1·50
(*b*) Mitochondrial pellet	1·65	0	1·65	1·45	0	1·45	1·40	0	1·40
Percentage of total nucleotide in pellet		85			88			94	

Reactivity of the pyridine nucleotides of flight-muscle mitochondria. Brief incubation (10 min.) of *Musca* mitochondria with succinate or pyruvate plus malate always increased the ratio DPNH + TPNH/DPN⁺ + TPN⁺; glutamate alone, glutamate plus malate and malate plus succinate were not as effective. α-Glycerophosphate sometimes increased the ratio (two experiments in five) but only once was the reduction of the coenzymes greater than with succinate (Table 2).

The losses of pyridine nucleotide during the incubations were usually small (approx. 20 %); the greatest loss was 44 %, with α-glycerophosphate as substrate.

Similar results (Table 2) were obtained with mitochondria isolated from *Lucilia* thoraces; reduction of the coenzymes was achieved most readily with succinate, less completely with pyruvate plus malate or α-glycerophosphate.

By resedimenting the mitochondria after incubation for 15 min. either without substrate or with succinate and then determining the coenzyme content of the whole suspension and of the particles isolated from it, it was shown (Table 3) that over 85 % of the pyridine nucleotides was retained by the mitochondria during this period of incubation in the saline medium, whether or not an oxidizable substrate was present (cf. Kaufman & Kaplan, 1960).

The rapid oxidation of the reduced nucleotides during centrifuging of the mitochondria (see Table 3) has been noted also with rat-liver mitochondria (Birt & Bartley, 1960*b*).

Effect of adenosine diphosphate on the oxidation of various substrates by flight-muscle mitochondria. The oxidation of glutamate and pyruvate plus malate was always increased by the addition of ADP, even when the preparations were made from flies up to 25 days old (Tables 4 and 10). The stimulation was not markedly affected by the concentration of mitochondria in the flask; thus in one experiment the respiratory-control ratios obtained initially with pyruvate plus malate and 1, 3 and 10 mg. of protein/flask were 1·8, 2·1 and 2·5 respectively; similar values were found with glutamate. However, the enhanced oxidation of pyruvate plus malate with ADP persisted for relatively longer periods of incubation at the lower concentrations of mitochondria (Table 4).

On the other hand, no stimulation by ADP of succinate oxidation occurred in any experiment; frequently, there was an inhibition of O_2 uptake, sometimes as great as 34 % (Table 5).

The effect of ADP on the oxidation of α-glycerophosphate was more complex and depended on (*a*) the concentration of mitochondria and (*b*) the age of the flies.

(*a*) Four direct comparisons of the effects of ADP made with suspensions containing different amounts of the same mitochondrial preparation showed no clear evidence of respiratory control when the amount of protein/flask was between 2 and 5 mg.; in the corresponding systems with 0·2–1·6 mg. of protein/flask, ADP increased the O_2 consumption by at least 70 % (Table 6, Expts. 1–4; see also Tables 4 and 5). Respiratory control and the more pronounced requirement for ADP in relatively dilute suspensions were also demonstrated when an ADP-regenerating system was used (Table 7). The stimulation of O_2 uptake was greatest after about 15 min., presumably because hydrolysis of added ATP during the preincubation provided some ADP initially. Because of the

Table 4. *Effect of concentration of mitochondrial suspension on rates of oxygen uptake*

(*a*) No ADP; (*b*) ADP, 10 mM. Incubations were at 25° in the Warburg apparatus; all substrates (50 mM) and ADP were added from the side arm after 10 min.

Concn. of mitochondrial protein (mg./flask)	Age of flies (days)	Time of incubation (min.)	Uptake of O_2 (μl./mg. of protein/hr.) Pyruvate plus malate (*a*)	Pyruvate plus malate (*b*)	Glutamate (*a*)	Glutamate (*b*)	Succinate (*a*)	Succinate (*b*)	α-Glycerophosphate (*a*)	α-Glycerophosphate (*b*)
10	4	0–5	35	73	2·6	7·2	67	70	—*	—
		15–20	15	8	4·0	9·4	75	67	0	0
3	6	0–5	22	56	1·2	6·8	84	81	120	125
		15–20	18	34	2·2	7·4	72	62	79	78
1·1	4	0–5	—	—	—	—	30	30	90	312
		10–20	25	45	1	5	35	33	215	428
0·6	4	0–5	Not estimated				52	50	210	435
		50–60					45	45	200	135

* Uptake of O_2 not measurable with this concentration of protein/flask; see text.

Table 5. *Effect of concentration of mitochondrial suspension in demonstration of respiratory control*

(*a*) and (*c*) No ADP; (*b*) and (*d*) ADP, 10 mM. Both substrates were initially 50 mM and, with the ADP, were added to the appropriate vessels at the beginning of the experiment without preincubation.

In Expts. A and C, after 25 min., ADP (10 mM) was added to (*a*), α-glycerophosphate (finally 25 mM) was added to (*b*). In Expt. B substrates and ADP were added after 25 min. Flies were 2 days old.

Time (min.)	Uptake of O_2 (μl./mg. of protein/hr.) A 3·9 mg. of protein/flask α-Glycerophosphate (*a*)	A 3·9 mg. of protein/flask α-Glycerophosphate (*b*)	A 3·9 mg. of protein/flask Succinate (*c*)	A 3·9 mg. of protein/flask Succinate (*d*)	B 3·9 mg. of protein/flask α-Glycerophosphate (*a*)	B 3·9 mg. of protein/flask α-Glycerophosphate (*b*)	B 3·9 mg. of protein/flask Succinate (*c*)	B 3·9 mg. of protein/flask Succinate (*d*)	C 0·39 mg. of protein/flask α-Glycerophosphate (*a*)	C 0·39 mg. of protein/flask α-Glycerophosphate (*b*)	C 0·39 mg. of protein/flask Succinate (*c*)	C 0·39 mg. of protein/flask Succinate (*d*)
0–5	170	150	79	52	0	0	0	0	40	550	0	0
15–20	15	29	79	55	0	0	0	0	40	420	30	30
30–35	7	170	93	73	200	230	57	62	220	380	30	30
40–45	0	94	87	73	36	26	70	80	300	380	30	30

Table 6. *Comparison of respiratory-control ratios obtained with different concentrations of the same mitochondrial suspensions*

Respiratory-control ratio

$$= \frac{O_2 \text{ uptake with } \alpha\text{-glycerophosphate plus ADP}}{O_2 \text{ uptake with } \alpha\text{-glycerophosphate alone}}$$

during first 5 min. of incubation.

Expt. no.	Age of flies (days)	Concn. of protein (mg./flask)	Respiratory-control ratio
1	8	4·8	0·91
		1·6	2·3
2	2	3·9	0·95
		0·4	13·7
3	5	2·3	1·3
		0·7	6·0
4	—	1·1	3·1
	2	0·6	4·0
		0·2	∞
5	14	4·3	0·83
		1·4	0·74
6	22	1·1	1·1
		0·4	1·3

high activity of the α-glycerophosphate oxidase of these mitochondria valid estimations of respiratory rates could not be made when the concentration of protein exceeded about 5 mg./flask, as the rate of O_2 uptake approached the limit imposed by the equipment used; in none of the experiments described above was the maximum respiratory rate greater than 75% of the experimentally determined capacity of the apparatus.

The sequence in which the particles were exposed to ADP and α-glycerophosphate also influenced the initial q_{O_2}. The O_2 uptake of mitochondria preincubated in α-glycerophosphate rose quickly (in less than 5 min.) to the maximum rate after the addition of ADP; addition of α-glycerophosphate to mitochondria preincubated in ADP produced a slower rise, reaching a maximum in 10–15 min. (Table 8). Also the respiratory rate with α-glycerophosphate alone or with ADP was higher after preincubation for 25 min. at 25° without substrate (Table 5).

Respiratory control was also demonstrated with α-glycerophosphate and suspensions of *Musca* mitochondria containing about 1 mg. of protein/flask. The initial respiration was stimulated approximately twofold during incubation in both the sucrose and (less consistently) the saline media.

(*b*) Even with an appropriate concentration of mitochondria, pronounced stimulations of respiration did not always follow the addition of ADP. As the age of flies increased it became more difficult to demonstrate respiratory control (Tables 6, 8 and 9). The loss of this requirement for ADP was not prevented by preparing and incubating the particles in a medium containing serum albumin (1%), which binds endogenous uncoupling agents (Hulsmann, Elliott & Slater, 1960), nor by incubation in a hypo-osmotic medium (0·12M with respect to sucrose).

Under those conditions, where no respiratory control was observed (i.e. with concentrated mitochondrial suspensions or with mitochondria from older flies), addition of ADP often caused an inhibition of O_2 uptake with α-glycerophosphate (see Tables 5 and 9; compare values for succinate oxidation, Table 5). In some of these experiments, ADP decreased the initial O_2 uptake by about 30%, but maintained respiration at a greater rate after 30–40 min.

Comparison of maximum rates of oxygen uptake with different substrates. As ADP is required for maximum rates of oxidation of all the substrates tested, except succinate, the comparisons of relative oxidase activities have been made between systems containing ADP.

1. There was no detectable endogenous respiration with or without ADP (Table 5).

2. Despite the impossibility of using a wide range of concentrations of mitochondria in suspensions oxidizing α-glycerophosphate, higher q_{O_2} values were obtained with this substrate and more dilute suspensions of the particles (Tables 6 and 7). The maximum rate recorded was 750 μl./mg. of protein/hr. (0·19 mg. of protein/flask); rates of 200–300 μl./mg. of protein/hr. were obtained with 1·0–1·5 mg. of protein/flask. Mitochondria from older flies usually oxidized α-glycerophosphate more slowly (compare Tables 8 and 10). The initial rates of O_2 uptake were the same with 100, 50 and 25 mM-α-glycerophosphate but were lower by about 80% with 5 mM; i.e. under the conditions used the oxidase system was saturated between 5 and 25 mM. Neither a decrease in the tonicity of the incubation medium nor addition of serum albumin (1%) influenced the rates of respiration greatly.

3. The rates of oxygen uptake with all the other substrates tested were lower than with α-glycerophosphate (Table 4). The maximum q_{O_2} with pyruvate plus malate was 73; with succinate, the maximum was 93 (mean value in seven experiments, 63). Very low q_{O_2} values were obtained with malate or glutamate (about 3 and 10 respectively). Combination of malate with succinate (without ADP) depressed the O_2 uptake to about one-quarter of that with succinate alone (Table 2, Expt. 2). Dilution of the mitochondria did not markedly influence the rates with any of these substrates, though there was some decrease in O_2 uptake as the flies aged (Table 10).

Thus the observed respiratory activities of the *Lucilia* mitochondria were similar to those ob-

Table 7. *Comparison of the effect of adenosine diphosphate and of an adenosine diphosphate-regenerating system on the oxidation of α-glycerophosphate*

Concn. of α-glycerophosphate, 50 mM; concn. of ADP, 10 mM. The ADP-generating system comprised, in the main compartment of the flasks, ATP (2 mM), hexokinase (0·1 mg.) and, in the side arm, glucose (final concn. 70 mM). In the control, hexokinase was replaced with serum albumin. In the experiments with the ADP-regenerating system, glucose and α-glycerophosphate were tipped in after 10 min. preincubation; in the other experiments, ADP and α-glycerophosphate were added together after 10 min. Readings were commenced at the time of tipping. Flies were 2 days old.

	Uptake of O_2 (μl./mg. of protein/hr.)									
	Concn. of protein (mg./flask)									
	1·1		0·55		0·19		1·1		0·19	
Time (min.)	–	+ADP	–	+ADP	–	+ADP	–	+Hexokinase	–	+Hexokinase
0–5	140	440	125	505	0	740	430	530	360	570
15–20	185	390	185	640	84	750	190	420	270	680
45–55	220	300	140	320	42	415	250	230	135	595

Table 8. *Effect of preincubation with adenosine diphosphate or α-glycerophosphate on respiratory rate*

(*a*) and (*b*): Preincubated without either ADP or α-glycerophosphate, then α-glycerophosphate was added to (*a*) and α-glycerophosphate plus ADP to (*b*). (*c*): Preincubated with ADP, α-glycerophosphate added. (*d*) and (*e*): Preincubated with α-glycerophosphate, then ADP was added to (*d*); nothing was added to (*e*). Concn. of α-glycerophosphate, 50 mM; conc. of ADP, 10 mM.

			Uptake of O_2 (μl./mg. of protein/hr.)				
Expt. no.	Age of flies (days)	Time of incubation (min.)	(*a*) Nothing	(*b*) Nothing	(*c*) ADP	(*d*) α-Glycerophosphate	(*e*) α-Glycerophosphate
1	4	(Preincubation)*	0	0	0	125	125
		5	20	110	160	295	210
		10	120	250	260	295	145
		15	160	250	260	250	165
		20	200	295	320	295	210
2	22	(Preincubation)*	0	—	0	60	—
		5	96	—	48	280	—
		10	145	—	180	240	—
		15	195	—	280	280	—
		20	240	—	280	280	—

* Values given are for the 5 min. period preceding the addition of the reactants.

Table 9. *Effect of age of flies on respiratory-control ratios with α-glycerophosphate*

Respiratory-control ratio

$$= \frac{O_2 \text{ uptake with } \alpha\text{-glycerophosphate plus ADP}}{O_2 \text{ uptake with } \alpha\text{-glycerophosphate alone}}$$

for 5 min. period preceding the times shown.

Age of flies (days)	Concn. of protein (mg./flask)	Respiratory-control ratios		
		5 min.	15 min.	30 min.
2	1·1	3·1	2·8	2·3
	0·6	6·5	7·5	2·4
4	1·1	3·5	2·1	0·9
10	1·4	1·9	1·1	1·1
20	1·8	0·9	0·8	0·9
	0·6	1·2	—	1·5
22	1·1	0·9	1·1	0·9
	0·4	2·0	1·1	1·1

tained with preparations from the housefly (Sacktor, 1958) and the locust (Bucher & Klingenberg, 1958), in that α-glycerophosphate was oxidized more readily than the other substrates tested. The particles were unable to oxidize pyruvate at the relatively high rates reported by Gregg, Heisler & Remmert (1959) and ATP did not affect the O_2 uptake with succinate (compare Azzone, Ernster & Klingenberg, 1960).

Stability of the respiratory rate. Whereas the rate of oxidation of α-glycerophosphate was much greater initially than that of any other substrate tested, the difference diminished during incubation since the O_2 uptake with α-glycerophosphate often declined and ceased. This occurred most readily when the ratio of the concentration of α-glycerophosphate to that of the mitochondria was relatively low (e.g. Table 5). A similar change did not

Table 10. *Effect of age of flies on respiratory-control ratios with various substrates*

Concn. of all substrates, 50 mM; conc. of ADP, 10 mM. R.C. ratio, respiratory-control ratio (see Table 9); q_{O_2} is the maximum rate of O_2 uptake observed, expressed as μl./mg. of protein/hr.

Age of flies (days)	Time of incubation (min.)	Pyruvate plus malate		Glutamate		α-Glycerophosphate	
		R.C. ratio	q_{O_2}	R.C. ratio	q_{O_2}	R.C. ratio	q_{O_2}
5	0–5	1·3	35	14	10	3·5	360
	15–30	2·0	—	3·0	—	1·6	—
	30–60	1·5	—	3·5	—	0·7	—
25	0–5	—	10·8	—	3·2	—	180
	15–30	1·6	—	3·0	—	1·2	—
	30–60	1·8	—	3·1	—	0·8	—

occur with any other substrate. When mitochondria were incubated with α-glycerophosphate (25 mM) and ADP until respiration ceased, addition of more substrate, but not of ADP, restored the original O_2 uptake (Table 5).

These results suggest that, in the manometric experiments, the cessation of respiration was due to the formation of such an amount of dihydroxyacetone phosphate that the ratio 'concentration of α-glycerophosphate/concentration of dihydroxyacetone phosphate' (calculated from the O_2 uptake) reached a value of about 3; in seven experiments, with initial concentrations of the substrate from 25 to 100 mM, O_2 uptake stopped when the value of this ratio was between 2·7 and 3·4. Estabrook & Sacktor (1958) have reported that dihydroxyacetone phosphate did not limit the oxidation of α-glycerophosphate (measured polarographically), even when the ratio was as low as 0·25. In view of the other differences in the respiratory behaviour of comparable preparations estimated by different techniques, this discrepancy is perhaps also due to differing experimental conditions.

When incubation was continued until respiration ceased, the total O_2 uptake by suspensions containing the same amounts of α-glycerophosphate and mitochondria was the same, despite differences in rates during the incubation. Thus in some experiments, the 'stabilizing' of the respiratory activity by the addition of ADP was probably due to an inhibition of O_2 uptake (see above) and the consequent delay in increasing the concentration of dihydroxyacetone phosphate to the value at which respiration stops.

DISCUSSION

The flight-muscle mitochondria of the two flies *Lucilia* and *Musca* contain similar total amounts of pyridine nucleotide. The coenzymes were almost completely oxidized in the freshly isolated particles and about 95% of the total was DPN^+. Slightly higher amounts of pyridine nucleotide have been found in the mitochondria of the locust (Klingenberg & Bucher, 1959) and the blowfly (Price & Lewis, 1959) and of some vertebrate muscles (e.g. for heart sarcosomes, see Holton, 1955). The high concentration of DPN relative to TPN in such particles (and in the entire thoraces of the flies; unpublished data) contrasts with the values obtained with mitochondria and homogenates of tissues such as liver and mammary glands of higher animals (e.g. see Birt & Bartley, 1960*b*; McLean, 1958), and is further support for the suggestion that the main function of DPN is in ATP production, whereas TPNH is used preferentially for reductive syntheses. As there is apparently some DPN-nucleosidase activity in the tissues (Table 1), the greater amount of coenzyme in the *Lucilia* preparations may reflect the shorter time required to prepare sufficient thoraces for the isolation of mitochondria for the experiments. The greater speed of preparation was also decisive in the choice of *Lucilia* for experiments on respiratory control.

Reduction of pyridine nucleotides by added substrate. All the substrates tested were capable of increasing the amount of reduced coenzyme in the mitochondria though only succinate was always effective. Even with this substrate the ratio $DPNH+TPNH/DPN^+ + TPN^+$ varied greatly in different experiments (compare Expt. nos. 2 and 4, Table 2). α-Glycerophosphate reduced the pyridine nucleotides less consistently (Table 2; cf. Bucher & Klingenberg, 1958). Two factors may account for this difference. First, it has been assumed that the reduction of DPN by succinate and α-glycerophosphate is due to a reversal of oxidative phosphorylation (Chance & Hollunger, 1957; Bucher & Klingenberg, 1958); alternatively, it has been proposed that an increased net reduction of the coenzymes by the malate produced from succinate is caused by an inhibition of DPNH oxidation during the simultaneous oxidation of the succinate (Birt & Bartley, 1960*c*). With α-glycerophosphate, only the endogenous substrate of the mitochondria would be available for the reduction and, as Klingenberg & Bucher (1959) have shown that the concentration of this material may decline rapidly in isolated

mitochondria, it is possible that the relatively greater variability in the extent of reduction with α-glycerophosphate is due to differences in the amount of DPN-reducing substances retained in different mitochondrial preparations.

Secondly, Klingenberg & Bucher (1959) have suggested that saline media are not well adapted to preserving mitochondrial structure and that reduction of pyridine nucleotides by α-glycerophosphate is a relatively labile function of the particles. Thus, whereas on this criterion the *Musca* mitochondria may be considered to be 'damaged', having lost their ability to reverse oxidative phosphorylation with α-glycerophosphate as substrate, on other criteria proposed by these authors (respiratory control with α-glycerophosphate, retention of coenzymes) they are 'undamaged'. Moreover, they have retained the ability to reduce pyridine nucleotides during the oxidation of succinate. While it is not possible with the data of this paper to decide which explanation of the results is correct, all the observations reported are compatible with the suggestion that the increase concentration of reduced coenzyme is a consequence of diminished opportunity for coenzyme oxidation.

The respiration of *Musca* mitochondria suspended in saline was not invariably increased by ADP. The attempt to increase the rate of this reduction by adding malate with succinate was vitiated by the pronounced inhibition of electron transport resulting, possibly by the rapid formation of oxaloacetate (Tyler, 1954).

Respiratory control by adenosine diphosphate. The results reported in this paper emphasize that with mitochondrial preparations the detection of respiratory control by ADP can depend completely on the conditions used in the experiment. An investigation of factors influencing the response of various mitochondrial oxidases to ADP has recently been published by Bellamy & Bartley (1960), who also concluded that different methods of estimating respiratory control can give widely differing results. The finding that, with mitochondria prepared from relatively young flies, the demonstration of a control of α-glycerophosphate oxidation by ADP was dependent on suitable dilution of the mitochondrial suspension, is in accord with their suggestion that the stimulation of uptake of oxygen by this phosphate acceptor may be facilitated by the equilibration of intramitochondrial solutes during shaking with the incubation medium. It is known that the mitochondria of another fly (*Calliphora*) contain ADP and ATP, at least some of which can be released into a buffered suspension medium (Price & Lewis, 1959). In the least concentrated suspensions of particles used in this work, the loss of ADP during equilibration in the incubation medium may be sufficient to lower the intramitochondrial concentration below that required for maximum oxidation of α-glycerophosphate. In more concentrated suspensions, where this would not occur, it is possible that the ADP added may limit the entry (or release) of substrates (Packer & Golder, 1960), and therefore depress the uptake of oxygen.

These two different effects of ADP may explain the stimulation or inhibition of uptake of oxygen observed under different conditions of incubation with ADP. Moreover, it is apparent that mitochondria preincubated in ADP or exposed simultaneously to ADP and α-glycerophosphate attain the maximum rate of oxygen uptake more slowly than those preincubated in α-glycerophosphate alone. No evidence was obtained for inhibition by ADP of the oxidation of any other substrate except succinate, and with this compound no stimulation was ever detected. Thus a change in the total extramitochondrial concentration of ADP may alter the structure and therefore the permeability of mitochondria in such a way as to produce a relatively selective exclusion of particular substrates, especially those which are themselves phosphorylated.

Slater & Lewis (1954) have shown that 2:4-dinitrophenol increased the respiration of *Calliphora* mitochondria oxidizing α-oxoglutarate only if the insects were relatively young, and Sacktor & Cochran (1958) reported that mature flies (3–7 days) do not exhibit respiratory control with α-glycerophosphate. With the experimental procedures described in this paper, respiratory control was always detectable with all substrates tested (except succinate) and flies up to 10 days old, and, in the initial stages of incubation, often with flies up to 1 month old. In general, however, the influence of ADP was less apparent with the older flies, both the dependence on ADP and the rates of oxidation of particular substrates declining. With pyruvate plus malate and glutamate, respiratory control was not lost in the mitochondria of older flies. The reason for the changes on aging is not known, but a decline in the rate of oxidation of certain compounds by liver mitochondria isolated from aging rats has been attributed to an increasing sensitivity of the particles to 'damage', resulting in inactivation of particular oxidases (Weinbach, 1959).

Bucher & Klingenberg (1958) have described a decrease in respiratory control during the aging of isolated locust mitochondria oxidizing α-glycerophosphate, but a similar change was not found during the relatively brief preincubation without substrate described in Table 2; indeed, in this experiment there was evidence for a slight increase in respiratory control after the preincubation (Table 5; cf. Bellamy & Bartley, 1960).

The results presented in this paper provide clear evidence that, given suitable experimental conditions and methods of estimation, the oxidation of α-glycerophosphate (and other substrates) by *Lucilia* mitochondria is controlled in part by the availability of ADP; and that pyridine nucleotides occur in the respiratory chain of two species of fly. They also underline some of the difficulties encountered in interpreting information obtained with isolated mitochondrial suspensions and the dependence of the observed behaviour of such systems on the conditions of preparation and incubation. In particular, it would seem to be premature to conclude that the control of the rate of respiration in the flight-muscle mitochondria of these insects is of a type radically different from that proposed for mitochondria from other organisms.

SUMMARY

1. The amounts and reactivity of the pyridine nucleotides of the flight-muscle mitochondria of the flies *Musca* and *Lucilia* have been estimated by a fluorimetric method and some factors influencing the rates of oxidation of various substrates have been investigated.

2. Particles freshly isolated from both flies contain about 2·5–3·0 μm-moles of total coenzymes/mg. of protein, almost all in the oxidized form. About 95% of the total is oxidized diphosphopyridine nucleotide.

3. Incubation with succinate, pyruvate plus malate and (less consistently) α-glycerophosphate increased the amount of reduced coenzyme. The maximum reduction observed was of 58% of the total, with succinate.

4. Not more than 15% of the pyridine nucleotides were lost from the particles in 15 min. incubation with or without substrate.

5. Adenosine diphosphate increased the respiration of dilute suspensions (about 0·4 mg. of protein/ml.) of mitochondria prepared from flies less than 2 weeks old and oxidizing tricarboxylic acid-cycle intermediates or α-glycerophosphate. Stimulations greater than tenfold were obtained.

6. With more concentrated suspensions (1·2–2·0 mg. of protein/ml.) or older flies, oxidation of α-glycerophosphate was no longer increased by adenosine diphosphate and was sometimes decreased.

7. q_{O_2} values (with adenosine diphosphate) were much higher for α-glycerophosphate (200–750 μl. of oxygen/mg. of protein/hr.) than for succinate (about 65), pyruvate plus malate (70), malate (3) or glutamate (10).

8. The results emphasize that the respiratory behaviour of mitochondrial preparations is influenced greatly by the techniques used for incubation and estimation.

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Direct Spectrophotometric Studies of the Kinetics of Oxidation and Reduction of Ubiquinone of Heart-Muscle Particles

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Spectrophotometric measurement of extinction changes associated with oxidation and reduction of the respiratory components of Keilin & Hartree heart-muscle particles has led to a clearer understanding of the reaction sequence and mechanism of action of these components (Chance, 1952*a*). In some cases it has been possible to distinguish between components whose reaction kinetics are either consistent or inconsistent with the rate of electron transfer through the system (Chance, 1952*a*, 1958). Although these methods have been applied to the ultraviolet region of the spectrum, for example to soluble enzymes (Chance & Harting, 1953; Harting & Chance, 1953), and to measurements of the 340 mμ band of reduced pyridine nucleotide in mitochondrial suspensions (Chance, 1954*a*, *b*; Chance & Williams, 1955*b*), their application to turbid heart-muscle preparations involves formidable technical difficulties. The arrangement of an 'end-on' photomultiplier placed very close to a cuvette containing dilute suspensions and the use of a high sensitivity to extinction changes (Chance, 1952*a*, 1954*a*, 1957*a*) have largely solved the problems discussed by Keilin & Hartree (1958), who encountered difficulties in the measurement of extinction changes of heart-muscle particles in the visible region of the spectrum with commercially available spectrophotometers.

In view of the intense interest in the possible role of ubiquinone (coenzyme Q) in electron transfer in various tissues and tissue fractions, more accurate and detailed information on the kinetics of its reduction and oxidation is needed. Up to this time, determinations of the ratio of oxidized to reduced forms of ubiquinone have been made by rapid stopping of the reaction either with cold methanol followed by a light-petroleum extraction (Pumphrey & Redfearn, 1960; Redfearn & Pumphrey, 1960) or by heat denaturation, followed by *cyclo*hexane extraction (Crane, Lester, Widmer & Hatefi, 1959; Hatefi, Lester, Crane & Widmer, 1959).

Direct spectrophotometric measurement of reactions of ubiquinone at 275 mμ requires light-sources of high intensity and stability. Further difficulties arise in providing optics of appropriate aperture and photosurfaces of adequate sensitivity, and in minimizing stray and scattered light. Also, extinction changes at 275 mμ occur rapidly upon addition of succinate to cyanide-treated heart-muscle particles. In this paper technical considerations involved in such measurements are described and ultraviolet-extinction changes are compared in both rate and extent with those computed from measurements of light-petroleum extracts of heart-muscle particles (Pumphrey & Redfearn, 1960).

Ubiquinone is the first component of heart-muscle particles to be studied simultaneously by direct spectrophotometry and by extraction from the particles. In heart-muscle and guinea-pig-kidney mitochondria assays of the changes of concentration of bound pyridine nucleotide (Chance & Williams, 1955*b*; Chance & Baltscheffsky, 1958*a*; Chance & Hollunger, 1960) have been correlated satisfactorily with chemical analyses (Klingenberg, Slenczka & Ritt, 1959).

We have shown that the ultraviolet-extinction changes measured in heart-muscle particles can be attributed to reduction of ubiquinone to the quinol. In terms of the kinetics and inhibitor-sensitivity of this reaction, our results confirm and expand those of Pumphrey & Redfearn (1959, 1960). A preliminary report of these studies is presented elsewhere (Chance & Redfearn, 1960).

EXPERIMENTAL

Materials

Horse heart is the most satisfactory material for chemical analysis because of its freedom from interfering pigments. Heart-muscle particles were obtained according to the general directions of Keilin & Hartree (1938, 1947) but with certain modifications, which are described in full in the appropriate references. The following terminology will be used: method M, particles prepared by grinding the mince in a mechanical mortar (Keilin & Hartree, 1938, 1947); method B, particles prepared by homogenizing the mince in a Waring Blendor (Chance, 1952*b*; elaborated by Pumphrey & Redfearn, 1960); method S, particles prepared from muscle mince washed with 0·9% NaCl soln. to remove cytochrome *c* (Tsou, 1952).

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Since the mechanism of action of ubiquinone does not appear to differ in the three preparations, a detailed description of their properties is not included.

Pure crystalline ubiquinone-50, m.p. 48·5°, was obtained from pig heart (Morton *et al.* 1958).

Phosphate buffers were prepared from KH_2PO_4 and Na_2HPO_4.

Methods

Spectrophotometry. Application of a double-beam spectrophotometer (Chance, 1954*a*) to the measurement of small extinction changes of cytochromes in heart-muscle mitochondria has been described by Chance & Baltscheffsky (1958*b*). The electronic output of the instrument represents the difference between extinction changes of the particle suspension at two wavelengths. Light-beams of the required wavelengths, obtained from a pair of Bausch and Lomb 250 mm. focus grating monochromators, are flickered alternately through the sample, and changes at one (reference) wavelength compensate for non-specific changes at the other (measurement) wavelength. To study the kinetics of reduction of ubiquinone, the measurement wavelength was set at 275 mμ and the reference wavelength at 300 mμ (275 – 300 mμ). To determine difference spectra, the reference wavelength was fixed at 300 mμ and various wavelengths in the range 250–310 mμ were used to measure the reduction of ubiquinone by addition of succinate to cyanide-treated heart-muscle particles. The spectrophotometer was illuminated with a 100 w Nestor arc lamp, the intensity of which was stabilized by a photoelectric control circuit in the same way as tungsten and mercury lamps (Chance, 1957*a*; Chance, Conrad & Legallais, 1958).

Accurate optical measurements at 275 mμ are difficult because of the sharp increase of extinction and scattering observed with heart-muscle particles at wavelengths below 320 mμ, and because extinction changes due to cytochromes in the Soret region may alter the amount of stray light falling upon the photocell.

The simplest test for the latter possibility is to use heart-muscle particles so highly concentrated that essentially no transmission occurs at 275 mμ. It is then possible to observe spurious responses at this wavelength due to cytochrome changes in the Soret region. In experimental tests, the double-beam spectrophotometer gives decreasing responses with increasing concentrations of particles and no spurious responses were observed. Co–Ni filters, transmitting between 250 and 310 mμ, were used to eliminate stray light, in determination of both extinction coefficients and difference spectra.

The extinction coefficient of ubiquinone is smaller without filters than with them, which is to be expected if some stray light passes through the monochromator. The significant fact is that the difference spectrum obtained without filters, and therefore in the presence of some stray light, agrees satisfactorily with that of ethanolic solutions of ubiquinone. This means that the absorption of stray light is not measurably changed by oxidation and reduction of cytochromes at other wavelengths, probably because the stray light has no sharp wavelength maxima. In addition, the largest changes due to cytochromes are in the region of the Soret band where both increases and decreases occur on reduction, and these changes apparently cancel out. This is important in determining difference spectra with the double-beam spectrophotometer and in recording rapid kinetic changes with the regenerative flow apparatus because optimum signal-to-noise ratios can be obtained only without filters. The double-beam apparatus has the advantage that it accurately measures increments of specific extinction in spite of appreciable amounts of 'constant' stray light.

Respiratory activity. Respiratory activity was measured by a vibrating platinum microelectrode (Chance & Williams, 1955*a*) or, at temperatures below 20°, by a stationary electrode in a rotating cell, a method developed by G. R. Williams. The latter assembly combines a calomel or Ag–AgCl reference electrode and a platinum microelectrode. These dip into a cell rotating at 120 rev./min. At the specified temperatures, the concentration of heart-muscle particles was high enough to avoid diffusion of O_2 into the cuvette (Chance & Williams, 1955*a*).

Kinetic analysis. The reduction of ubiquinone was initiated by stirring succinate rapidly into an open cuvette of 1 cm. path, containing 1–2·5 ml. of heart-muscle particles. The mixing time was < 1 sec. (Chance, 1950). Experiments were conducted at 25° or at 10° (Table 3). Assays at the lower temperature are believed to be more reliable since the reduction of ubiquinone may be so rapid in active preparations at 25° that it is incorrectly recorded.

Methods of determining (i) the rates of oxidation and reduction of ubiquinone and (ii) the steady-state oxidation–reduction levels of endogenous ubiquinone are described elsewhere (Pumphrey & Redfearn, 1960; Redfearn & Pumphrey, 1960).

Determination of exogenous ubiquinone. A mixture of 0·2 ml. of heart-muscle particles (method B, 34·7 mg. of protein/ml.), 0·8 ml. of phosphate buffer (0·1 M, pH 7·4), 0·1 ml. of KCN (0·06 M) and 0·05 ml. of ubiquinone in ethanol was incubated at 26° with 0·2 ml. of 0·3 M-sodium succinate. The reaction was stopped and ubiquinone was extracted and assayed as described by Pumphrey & Redfearn (1960).

Kinetics of reduction of exogenous ubiquinone. The reaction mixture consisted of heart-muscle particles, buffer and KCN as above, together with 0·05 ml. of ethanolic ubiquinone (3·5 mM). The reaction was started by the addition of 0·2 ml. of 0·3 M-succinate. The reaction was stopped and the residual ubiquinone assayed as above.

Estimation of protein. The heart-muscle particles were diluted to between 1 and 10 mg. of protein/ml. and 1 ml. of the diluted particles was mixed with 0·5 ml. of water, 1·5 ml. of biuret reagent (Gornall, Bardawill & David, 1949) and 2 drops of 40 % (w/v) sodium cholate. The mixture was incubated for 30 min. at room temperature, and the extinction at 540 mμ then read in a spectrophotometer. The protein concentration in the diluted particles is given by $E_{540\,m\mu}$/0·096 mg. of protein/ml.

In the experiments of Figs. 1, 2*a* and 3, the simple relationship, fat-free dry weight $= 1{\cdot}3 \times E_{500\,m\mu}$ (Chance, 1952*b*), was used.

RESULTS

Optical data

A criterion for the measurement of a valid optical response. Addition of succinate to heart-muscle particles gives a readily measurable signal due to fumarate production (Chance, 1952*a*, *b*). A typical example of such an experiment is shown in Fig. 1.

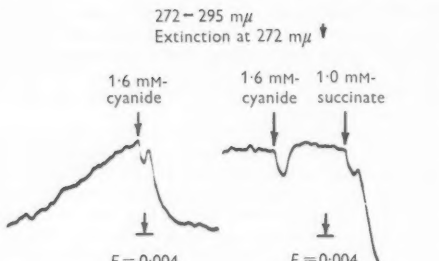

Fig. 1. Kinetics of u.v.-extinction changes in heart-muscle particles. *Left.* Treated with succinate and then with cyanide. M-Succinate was added to a final concentration of 8 mM. The upward slope of the trace indicates increasing extinction, measured at 272 – 295 mμ, due to the formation of fumarate. M-Cyanide was added to a final concentration of 1·6 mM; after a small artifact caused by stirring, there was decreased extinction at 272 mμ (downward slope of the trace). *Right.* Particles were pretreated with M-cyanide to a final concentration of 1 6 mM. After an artifact caused by stirring, no net change of extinction occurred. The addition of M-succinate to a final concentration of 1·0 mM was followed by a large decrease of extinction, measured at 272 – 295 mμ. Particles (method B, 0·5 mg. of protein/ml.) in 0·1 M-phosphate buffer, pH 7·2; temp. 26°; Co–Ni filter. (Expt. 956*b*.)

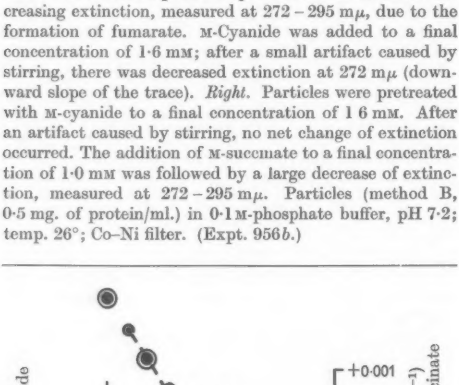

Fig. 2*a*. Comparison of the magnitude of extinction changes caused by adding cyanide (○) to succinate-treated heart-muscle particles (0·42 mg. of protein/ml.) with the rate of extinction changes caused by adding succinate (●) and with the spectrum of pure fumarate (⊙). The left-hand scale of the ordinate corresponds to changes with cyanide for an optical path of 1 cm., the right-hand scale to the rate of change in extinction with succinate and to the extinction of pure fumarate. The peak observed in the presence of cyanide (~ 280 mμ) is close to the expected location for the extinction peak of ubiquinone. (Expt. 955.)

On the left the rise of the trace, corresponding to increasing extinction (272 – 295 mμ), indicates production of fumarate. Addition of cyanide causes an abrupt downward deflexion, indicating a decrease of $E_{272\,m\mu}$. On the right the preparation is pretreated with cyanide, which causes no extinction change other than that due to the stirring artifact (small dip and rise of the trace). On addition of succinate, there is an abrupt downward deflexion, which exceeds considerably that observed on the addition of cyanide (left). This suggests that the reaction is incomplete in the presence of succinate only. To determine the wavelength dependence of these effects the latter experiment was repeated at a succession of wavelengths (Fig. 2*a*, solid circles). Since concentrations of heart-muscle particles and of succinate were not varied in these experiments, the slope of the trace representing fumarate production should be proportional to the extinction coefficient of fumarate at the wavelengths used. To verify this, the rate of extinction change in the presence of succinate is plotted as a function of wavelength in the spectral region 250–300 mμ (solid circles). The extinction rises at shorter wavelengths in a manner characteristic of the extinction spectrum of fumarate. A quantitative test of the shape of the fumarate spectrum is afforded by the circled points, which represent the spectrum of fumarate in solution (Racker, 1950). This result supports the conclusion that the shape of u.v. spectra can be measured without measurable effects from light-scattering and stray-light errors, and verifies that the oxidase activity of heart-muscle particles can be measured by the rate and extent of fumarate production (Chance, 1952*b*).

The difference spectrum of extinction changes that occur upon adding cyanide to succinate-treated heart-muscle particles is represented by the open circles of Fig. 2*a*. Values of the ordinate here correspond to the change of extinction for a 1 cm. optical path. The peak just below 280 mμ is near the expected location for the peak of ubiquinone. Since this peak disappears upon reduction, the values of the ordinate are given a negative sign.

Activity criterion and effect of dilution of heart-muscle particles. It was desirable to have a standard value relating rate of fumarate production to concentration of material reducible by cyanide in succinate-treated heart-muscle particles. The left trace of Fig. 1 indicates the nature of this test, which consists merely of determining the quotient of the slope of the fumarate trace and the extinction change occurring on addition of cyanide to succinate-treated particles. For a given preparation this quotient should be constant, since it has the dimensions of enzymic activity (rate of produc-

tion of product divided by amount of enzyme, i.e. sec.$^{-1}$). Table 1 summarizes these values for a variety of dilutions of a heart-muscle suspension. Experimentally, the method is sufficiently sensitive for satisfactory measurements with heart-muscle particles diluted 167 times, at which a value of 210 is obtained. With dilutions of 130- and 86-fold slightly lower values are obtained, but, with a 60-fold dilution, a large discrepancy in the ratio is observed. However, dilutions between 167- and 86-fold (0·4–0·7 mg. of fat-free dry wt./ml.) are satisfactory for experimental studies at ~272–295 mμ. The constancy of these values does not necessarily imply that extinction changes due to production of fumarate and reduction of ubiquinone are individually measured with accuracy, but rather that proportional errors may be made.

Comparison of difference spectra of ultraviolet-extinction changes with those of ubiquinone extracted with light petroleum. In Fig. 1 (left) extinction change observed on adding cyanide could conceivably have been due to a stray-light effect, light-leakage in the visible region being modified by reduction of cytochrome that occurs when cyanide is added to succinate-treated heart-muscle particles. However, since reduced forms of cytochromes absorb more light than oxidized forms, it is unlikely that reduction of cytochromes is responsible for the apparent decrease of extinction. Nevertheless it was considered important to make a detailed investigation of the nature of the u.v.-extinction band that disappears on reduction of the components in heart-muscle particles. For this reason we have repeated the experiment of Fig. 2*a* (solid trace) without Co–Ni filters (Fig. 2*b*) and have superimposed upon it the spectrum of an ethanolic solution of ubiquinone extracted from identical particles. These two spectra are normalized so that their maxima are superimposed. Although the shapes are similar, there is some displacement toward longer wavelengths of the difference spectrum for heart-muscle particles. There are three possible explanations for this. The first is that ethanol was the solvent for the difference spectrum of extracted ubiquinone, whereas the medium for endogenous ubiquinone might in effect be water or lipid. The second is that a certain amount of 'pulling' of the spectrum of endogenous ubiquinone towards longer wavelengths might be expected because (*a*) in this region extinction by protein components of heart-muscle particles increases rapidly with decreasing wavelengths and (*b*) there is increased light-scattering at shorter wavelengths. The third is that extinction changes due to haemoproteins might contribute on the long-wavelength side of the extinction band. Of these possibilities, the last is the most improbable: cytochrome *c*, for which quantitative data are available (Margoliash & Frohwirt, 1959), shows the same changes of extinction upon oxidation and reduction at 275 mμ as at 300 mμ. Between these two wavelengths, there would be a relative diminution of extinction in the transition from oxidized to reduced states, so that, at least for cytochrome *c*, any influence from the δ band is unlikely. We consider the first and second explanations for the shifted extinction peak more likely.

Table 1. *Effect of dilution of heart-muscle particles on extinction changes of ubiquinone* (*Expt.* 955*c*)

Heart-muscle particles (method B), 63 mg. of fat-free dry wt./ml., were used at the dilutions indicated; 0·1 M-phosphate, pH 7·8. The reaction was initiated with M-succinate to a final concentration of 8 mM. After the rate of fumarate production had been measured, M-cyanide was added to a final concentration of 1·6 mM, causing the reduction of ubiquinone from the steady state to the fully reduced state. The ratio of the rate of fumarate production to the extinction change of ubiquinone in response to the addition of cyanide is termed the 'activity value'.

Dilution	167	130	86	60
Concn. (mg./ml.)	0·4	0·5	0·7	1·0
Activity value (sec.$^{-1}$)	210	180	180	320

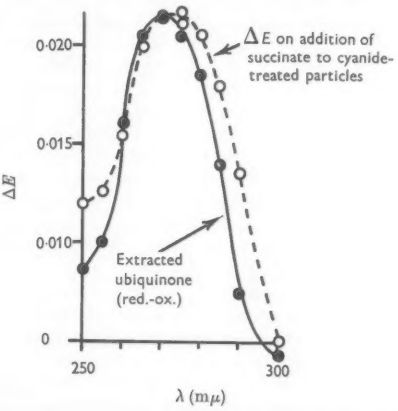

Fig. 2*b*. Superposition of extinction changes observed on addition of succinate to cyanide-treated heart-muscle particles (0·8 mg. of protein/ml.) (○) and those recorded for an ethanolic solution of ubiquinone extracted from identical particles (●). The latter spectrum was obtained by the indirect method described by Pumphrey & Redfearn (1960), the former by the direct method. Heart-muscle particles (method B) were diluted in 0·15 M-phosphate buffer (pH 7·8) for 30 sec.; M-cyanide was added to a final concentration of 1·2 mM; M-succinate was added to a final concentration of 4 mM. Temp. 26°; Co–Ni filters were not used. (Expt. 987*a*.)

The first is particularly applicable because ubiquinone absorbs at a longer wavelength in aqueous suspension than it does in ethanol (B. Chance & E. R. Redfearn, 1959, unpublished work). In fact, it is possible that in the heart-muscle particles ubiquinone absorbs at a wavelength differing from that of ubiquinone in any known solvent.

Extinction coefficient of ubiquinone. The extinction change observed on reducing ubiquinone with succinate in the presence of cyanide in heart-muscle particles is less than that computed from the chemically determined ubiquinone content of particles having the same protein concentration (Table 2). Further experiments were therefore made to determine the effective value of the extinction coefficient of a solution of ubiquinone under both sets of experimental conditions. According to Pumphrey & Redfearn (1960), the change of molar extinction coefficient corresponding to the reduction of ubiquinone dissolved in ethanol and measured at 275 – 300 mμ is 13 cm.$^{-1}$ mM^{-1}. This value was calculated from the absolute absorption spectra of ubiquinone and the quinol. A value of 13 is also obtained in the apparatus we use, but if ubiquinone is dispersed in water the extinction coefficient is less (Table 2). If an ethanolic solution of ubiquinone is added to heart-muscle particles and Co–Ni filters are used, the extinction coefficient is between 5 and 8 cm.$^{-1}$ mM^{-1}. Under these conditions, the extinction change due to reduction of endogenous ubiquinone divided by the concentration of ubiquinone obtained on analysis of an extract gives a coefficient of ~6 cm.$^{-1}$ mM^{-1} for a 1 cm. path cuvette and the particular optical geometry described under Methods. Since there are uncertainties in the completeness of the indirect extraction method and in optical calibration obtained by adding a suspension of ubiquinone to the heart-muscle particles, we have arbitrarily chosen a value of 10 cm.$^{-1}$ mM^{-1} for rate calculations in this paper.

Extinction coefficient of fumarate. As a further check upon the accuracy of the spectrophotometric method, the extinction coefficient of fumarate was determined by quantitative reduction of the oxygen dissolved in the air-saturated buffer solution of heart-muscle particles with formation of a corresponding concentration of fumarate. The fumarase activity of these particles is negligible compared with the succinic-oxidase activity. For the reaction to be complete within a reasonable time, a relatively high concentration of particles is used (0·5 mg./ml.), and this requires an optical path of 2 mm. rather than 1 cm. for accurate measurement of the large extinction change. A typical record appears in Fig. 3, where the extinction change corresponding to the formation of fumarate is 0·0356. Taking the solubility of oxygen in phosphate buffer to be 240 μM (Chance & Williams, 1955*a*), the extinction coefficient is calculated to be 0·37 cm.$^{-1}$ mM^{-1}.

Titrimetric and inhibition data

Effect of succinate concentration on the reduction of ubiquinone. In some heart-muscle-particle preparations, the rate of reduction of ubiquinone on adding succinate was sufficiently slow that it could be measured at 26° (cf. Figs. 1, 4 and 5). Fig. 4 illustrates the effect of two concentrations of succinate on the rate and extent of reduction of ubiquinone in cyanide-treated heart-muscle particles. A straight line is obtained when reciprocals of the rates are plotted against reciprocals of succinate concentration within the range 0·030–1·00 mM-succinate. The succinate affinity for ubiquinone reduction is 0·5 mM and that for half-maximal respiration, as

Table 2. *Millimolar extinction coefficients (Expt. 990)*

The millimolar extinction coefficients of endogenous and added ubiquinone were determined under the following conditions: particles (method B), were diluted to 0·8 mg. of protein/ml. in 0·1 M-phosphate, pH 7·2. They were treated at 26° with M-cyanide to a final concentration of 1·2 mM and then with M-succinate to a final concentration of 4 mM. The extinction change was measured at 275 – 300 mμ, with Co–Ni filters. Ethanolic ubiquinone (0·5 mM) was added to a final concentration of 1·85 μM and the change measured again. Extinction coefficients were determined as described in the text.

	Solvent		Heart-muscle particles
	Ethanol	Water	
$\Delta\epsilon$	13	9	6 (endogenous) 5–9 (added)

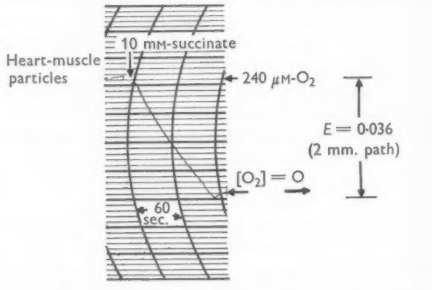

Fig. 3. Determination of a molar extinction coefficient for fumarate in succinate-treated heart-muscle particles. The downward deflexion of the trace indicates fumarate production and an increase of extinction at 275 – 300 mμ. Particles (method B, 0·5 mg. of protein/ml.) in 0·1 M-phosphate buffer were equilibrated with air at 26°. (Expt. 96–95*b*.)

measured by the platinum microelectrode, is also 0·5 mM (Chance, 1952*b*).

Effect of antimycin A. Pumphrey & Redfearn (1960) found that both the rate and the extent of reduction of ubiquinone in heart-muscle particles were unaffected by antimycin A. Since this is a crucial observation in interpreting the locus of action of ubiquinone, we have repeated the experiment spectrophotometrically (Fig. 5). When 4 mM-succinate is added to cyanide-treated heart-muscle particles (left), the spectrophotometric trace shows an upward deflexion, corresponding to a decrease in extinction at 275 – 300 mμ. If, instead of cyanide, sufficient antimycin A is added to inhibit respiration completely (right), subsequent addition of succinate also causes an upward deflexion. In this case, the rate of change of extinction is only 4 % less than that observed with

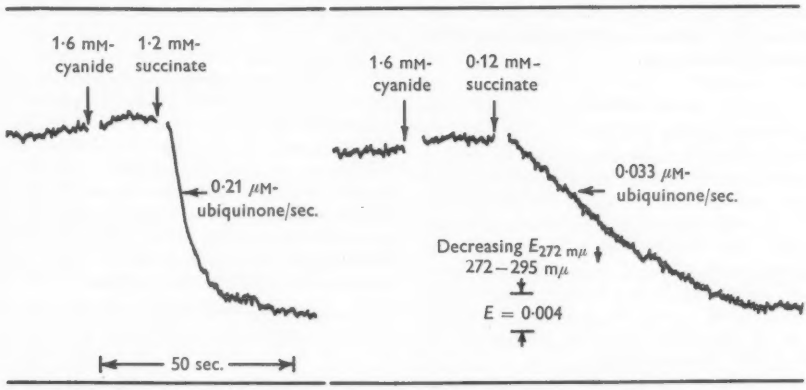

Fig. 4. Effect of succinate concentration on the rate of extinction change of heart-muscle particles, measured at 272 – 295 mμ. The particles were pretreated with M-cyanide to a final concentration of 1·6 mM. Succinate was added to a final concentration of 1·2 mM (left) and 0·12 mM (right). The experimental conditions were identical with those of Fig. 1. (Expt. 956.)

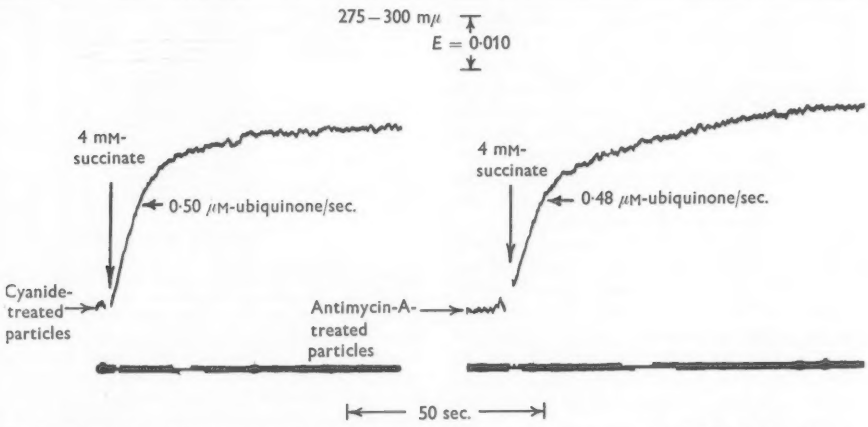

Fig. 5. Comparison of the kinetics and extent of reduction of ubiquinone in cyanide-inhibited and antimycin-inhibited heart-muscle particles. *Left.* Particles were pretreated for ~30 sec. with M-cyanide to a final concentration of 1·2 mM and then with M-succinate to a final concentration of 4 mM. The rise of the trace indicates decreased extinction at 275 – 300 mμ, and the reduction of ubiquinone. *Right.* Particles were pretreated for ~30 sec. with antimycin A (1 mg. dissolved in ethanol) to a final concentration of 2 μM and then with M-succinate to a final concentration of 4 mM. The experimental conditions were as in Fig. 2*b*. (Expt. 990*b*-11, 12.)

cyanide-treated particles. The equality, within experimental error, of the changes of extinction in the two experiments is of particular interest. It appears that all the u.v.-absorbing material can be reduced as effectively in the presence of antimycin A as in the presence of cyanide. Chemical assays also indicate that all the petroleum-extractable ubiquinone is reduced under these conditions (Pumphrey & Redfearn, 1960). Thus it seems reasonable to conclude that the quinone acts on the substrate side of the antimycin-A-sensitive point and that no detectable amount operates between cytochrome c_1 and oxygen.

Kinetic data

Nature of the reaction kinetics. Examination of the reduction kinetics, as illustrated particularly in Figs. 4 and 5, shows that the reaction is zero order for the first two-thirds of its course. No measurable 'jump' in the reaction kinetics occurs on addition of succinate. For this reason, rates of reduction are computed on the basis of the slope of the linear portion of the traces. It is a matter of some concern that chemical extraction gives results which appear to fit first-order kinetics (Pumphrey & Redfearn, 1960; Chance, 1960).

Effect of adding ubiquinone to heart-muscle particles. In view of the considerable interest in reactivation of electron transfer in solvent-extracted heart-muscle particles (Green, 1959), we have determined the spectrophotometric response of particles to added solutions of ubiquinone. Fig. 6 illustrates the reduction of endogenous ubiquinone by addition of succinate to cyanide-treated particles. When reduction is practically complete, ethanolic ubiquinone is added to a final concentration of 1·85 μM. The abrupt rise of the trace is due to extinction of the added oxidized ubiquinone, but the ensuing horizontal trace shows that this ubiquinone remains oxidized for at least 30 sec., whereas the endogenous ubiquinone was reduced in a few seconds.

This result was confirmed by chemical studies in which the time scale was extended beyond 30 sec. Only 25 % of the total ubiquinone was reduced in 1 min., and less than 50 % in 5 min. The concentration of the added ubiquinone (134 μM) was about six times that of the endogenous ubiquinone and therefore represented a more favourable condition for a rapid reduction reaction than in the spectrophotometric study of Fig. 6.

It is thus apparent that there is little relationship between the activities of the respiratory chain with respect to endogenous ubiquinone and to added ubiquinone.

Kinetics of reduction of endogenous ubiquinone: comparison of indirect chemical and direct spectroscopic methods. In several experiments spectroscopic and chemical methods were compared in the same preparations under nearly identical conditions. The ubiquinone-reductase activities are compared with the succinic-oxidase activities in Table 3. The values in the Table indicate, as was found previously (Pumphrey & Redfearn, 1959; Redfearn & Pumphrey, 1960), that the rate of reduction of ubiquinone by succinate is only a fraction of the succinic-oxidase rate. The validity of this conclusion is considered in more detail in the Discussion.

Comparison of rate of fumarate production with rate of ubiquinone reduction. Experiments such as those of Fig. 1 give rates of fumarate production and ubiquinone reduction under identical conditions. In Fig. 1 the ratio of the rate of extinction change of ubiquinone to that of fumarate is 7. An experiment in which an attempt was made to obtain a higher accuracy is illustrated by Fig. 7. Here the temperature was 8° and the optical path was 2 mm. Under these conditions, a higher concentration of heart-muscle particles was permissible and the rate

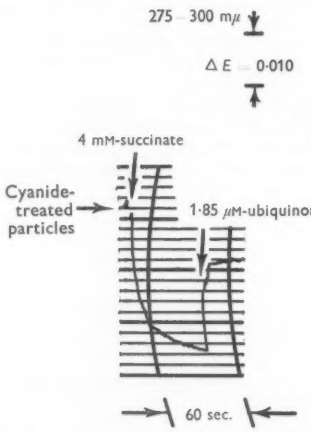

Fig. 6. Comparison of the rates of reduction of endogenous and exogenous ubiquinone in heart-muscle particles. The particles were pretreated with M-cyanide for 30 sec. to a final concentration of 1·2 mM and then with M-succinate to a final concentration of 4 mM. The reduction of ubiquinone is indicated by the downward deflexion of the spectrophotometric trace (decreased extinction at 275 – 300 mμ). When the reaction was substantially complete, 0·5 ml. of ethanolic ubiquinone was added to a final concentration of 1·85 μM. The upward deflexion of the trace corresponds to increased extinction of oxidized ubiquinone. The horizontal portion of the trace indicates that ubiquinone was not significantly reduced. The Co–Ni filter was used; conditions otherwise were as in Fig. 2*b*. (Expt. 990*b*.)

Table 3. *Succinate–ubiquinone reductase rates in heart-muscle particles*

Details of the spectrophotometric experiments are given in the Figures, legends and text. The experimental data have been multiplied by the dilution factor for the particular experiment so that they will be comparable with the results calculated from the extraction method. The values can be converted into μmoles/mg./hr. by multiplying by 3·6 and dividing by the protein concentration. Particle suspensions were diluted 30–40-fold: activities are given for undiluted preparations. (Expts. 994, 996 and 998.)

				Rate of reduction by succinate				Rate of oxidation of succinate			
				By extraction method		By spectrophotometric method		O_2 electrode method			Ubiquinone reductase / Succinic oxidase
Method	Concn. of protein (mg./ml.)	[Ubiquinone] (μmoles/g. of protein)	Age of prep. (days)	(μmoles/l./sec.)	Temp.	(μmoles/l./sec.)	Temp.	(μmoles/l./sec.)	Temp.	
M	34·5	3·5	1	—	—	—	—	364	25°	—
M	34·5	3·5	5	7·2	0°	9·1	7°	31	7	0·29
M	34·5	3·5	7	8·1	7	10	7	15·5	8·5	0·58
B	29·6	3·5	12	22	7	27	9·5	73	7	0·34
M	34·2	3·3	2	10·4	7	7·4	7	40	8·5	0·22

of production of fumarate was rapid even at the low temperature. The ratio of rates of extinction changes was now 7·5 or 9·0 depending upon the order of addition of succinate and cyanide.

Steady state. Fig. 1 clearly indicates that the extinction change obtained on adding succinate to cyanide-treated particles exceeds that obtained on adding cyanide to succinate-treated particles. If this is considered evidence of partial reduction of ubiquinone in the steady state of succinate utilization in heart-muscle particles, the percentage reduction of ubiquinone under these conditions was ~30% (Table 4). In general, the extent of reduction found chemically considerably exceeded that measured optically, at both room and low temperatures. The cause of this discrepancy is not known. However, in a cytochrome *c*-deficient preparation (method S), the values are in reasonable agreement.

DISCUSSION

The fundamental result of these experiments is that a decrease of u.v. extinction occurs upon addition of succinate to either cyanide-free or cyanide-inhibited heart-muscle particles but that the decrease is considerably greater in the presence of the inhibitor. The succinate concentration required for half-maximal change of extinction is roughly the same as that required for half-maximal respiratory activity. Upon addition of oxygen to anaerobic, succinate-reduced heart-muscle particles, u.v. extinction increases rapidly, later subsiding at a rate consistent with the rate of oxygen exhaustion by the system. Although these observations suggest that a component of the respiratory chain is being studied (Green, 1959), a more detailed investigation is required if this point is to be established.

A quantitative study of the kinetics of the change of u.v. extinction necessitates (i) identification of the substance involved, (ii) an estimate of its molar extinction coefficient, (iii) determination of its site of action in the respiratory chain, and (iv) comparison of the rates of its oxidation and reduction with the rate of electron transfer in the cytochrome chain.

(i) Interpretation of the u.v.-extinction change as a reduction of ubiquinone depends upon satisfactory agreement between the change caused by succinate addition to intact heart-muscle particles described in this paper and that caused by addition of sodium borohydride to a light-petroleum extract of ubiquinone from identical particles (Pumphrey & Redfearn, 1960).

The possibility might be considered that ubiquinone is reduced to a free radical or semiquinone (Michaelis & Schubert, 1938) which could mediate

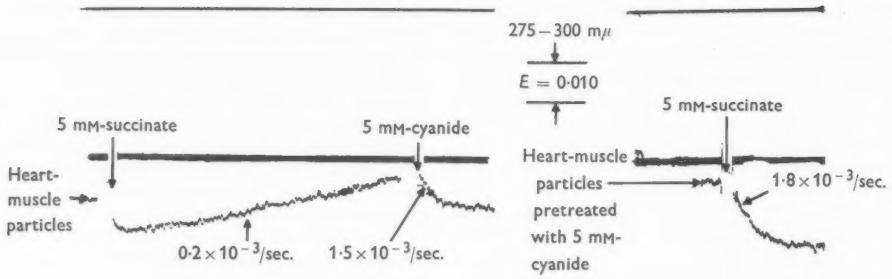

Fig. 7. Comparison of the rates of change of extinction in heart-muscle particles caused by the production of fumarate (*a*) in the presence of succinate (upward slope of the trace) with the rate of reduction of ubiquinone after cyanide addition or (*b*) with the rate of reduction of ubiquinone upon addition of succinate to cyanide-treated particles. Particles (method B, 0·39 mg. of protein/ml.) in 0·1 M-phosphate buffer, pH 7·4; temp. 8°. (Expt. 95.)

Table 4. *Steady-state oxidation–reduction levels of endogenous ubiquinone*

Experiments were carried out as described in the text and Figure legends. For the spectrophotometric experiments, the percentage reduction was calculated from the ratio of the recorder deflexions obtained on adding succinate to cyanide-free heart-muscle particles to the sum of this deflexion and that obtained on adding succinate to cyanide-inhibited particles. For the extraction method, the values were obtained by adding first succinate and then cyanide to heart-muscle particles.

Particle preparation	Age of prep. (days)	Temp.	Steady-state reduced ubiquinone (%)	
			Extraction method	Spectrophotometric method
B	8	9·5°	35	13
B	2	26	50	—
M	2	7	47	29
M	3	7	55	25
M	3	9·5	—	35
S	5	26	73	88

between two-electron steps in flavins and one-electron steps in cytochromes (Chance & Williams, 1956). However, the close correspondence of the difference spectra of solutions of ubiquinone with those of heart-muscle particles provides no evidence for such an intermediate. These observations also make it unlikely that a spectroscopically distinct ubiquinone–phosphate compound is present at measurable concentrations in the non-phosphorylating particles.

(ii) Although a precise determination of the molar extinction coefficient of ubiquinone in the heart-muscle particles is not yet possible, an approximate value can be suggested. The extinction coefficients measured in ethanol or in water in the double-beam spectrophotometer agree with those measured in the Beckman DU spectrophotometer in ethanol or in water. However, the extinction coefficient in water is much less than that in ethanol and agrees with the coefficient computed from addition of known concentrations of ubiquinone to a water suspension of heart-muscle particles. This value in turn agrees with the extinction coefficient of endogenous ubiquinone calculated from the quotient of the extinction change, measured by addition of succinate and cyanide to the particles, and the concentration obtained by analysis of an extract of the particles. Although the coefficient for endogenous ubiquinone may be as low as ~ 6 cm.$^{-1}$ mM^{-1} we have concluded that a value of 10 cm.$^{-1}$ mM^{-1} represents as good a value as the data warrant at the present time. It is obvious that the rates of reduction of ubiquinone given here would be increased

proportionally if 6 cm.$^{-1}$ mM^{-1} were subsequently found to be a more nearly correct value for the extinction coefficient.

(iii) Experiments with antimycin A indicate that ubiquinone is on the substrate side of the antimycin-sensitive point in the respiratory chain. The extinction change at 275 mμ that accompanies succinate reduction in the presence of cyanide is no greater than in the presence of antimycin A. Nor does addition of cyanide to a system blocked with antimycin A and reduced with succinate cause any further increase of extinction (B. Chance, 1959, unpublished work). These results suggest, contrary to the postulates of Martius (1955) on the general function of quinones and those of Green (1959) on the role of ubiquinone in particular, that no measurable amount of ubiquinone participates between the antimycin-sensitive point and oxygen. These results confirm previous titrations of the respiratory chain with reduced DPN (Chance, 1957*b*). Those titrations showed that ubiquinone does not act as an intercytochrome carrier but the possibility that ubiquinone participates near the substrate end of the chain (Slater, 1957) was not eliminated. The remarkable inactivity of heart-muscle particles in the reduction of added ubiquinone suggests that high reductase activities towards exogenous ubiquinone can be obtained only by structural alteration of the particles to enhance accessibility of the reductase (Bendall & de Duve, 1960).

(iv) Correlations of ubiquinone and oxygen reduction activities have been made on various preparations. Results observed with our spectrophotometric or 'direct' method can now be compared with those reported by Redfearn & Pumphrey (1960) for the chemical extraction or 'indirect' method. Since none of the assays given here was made in the presence of added cytochrome *c*, the comparison is limited to data in Table 3 of Redfearn & Pumphrey (1960). It is apparent that in the absence of added cytochrome *c*, the succinic-oxidase values in this paper, measured at 25°, are considerably greater than those measured at 21° by Redfearn & Pumphrey. For example, an experiment at room temperature (Table 3, first row), suitably corrected, gives 38 μmoles/mg. of protein/hr., whereas Redfearn & Pumphrey report values between 3·5 and 6·9 μmoles/mg. of protein/hr. Thus the values in this paper pertain to much more active preparations.

An activity of 38 μmoles/mg. of protein/hr. corresponds to 11 μmoles/g./sec. With a ubiquinone concentration of 4 μmoles/g., this amount would be turned over in 4/11 = 0·36 sec. at 25°, provided it were in the main pathway of electron transfer. This time is too short for manual mixing, i.e. indirect, methods at room temperature. It is important to note that dilution of the heart-muscle particles will not increase the time available for the measurement of ubiquinone reduction because this is a bound enzyme and its turnover time is independent of dilution (Chance, 1952*a*). For these reasons Table 3 includes four measurements of ubiquinone-reductase activities at 7–9·5° where the time, as calculated above, ranges from 8 to 1·4 sec., the latter being just satisfactory.

The ubiquinone-reductase activities are computed from the initial slopes of the reaction kinetics as measured by the indirect chemical extraction method or by the direct spectroscopic method with a value of $\Delta\epsilon_{275-300\,m\mu}$ of 10 cm.$^{-1}$ mM^{-1}. The three values obtained in the temperature range 7–9·5° agree to within 20%, which is considered highly satisfactory for present purposes.

The ratios of the ubiquinone-reductase activities (means of direct and indirect methods) to the succinic-oxidase activities measured polarographically have a mean value of 0·36. Thus the ubiquinone-reductase activity would appear to be about one-third of the succinic-oxidase activity.

The agreement between determinations of the steady-state reduction of ubiquinone by indirect and direct measurements is not as close as desirable, the latter method giving smaller values. Direct measurement of steady-state reduction was made approximately 30 sec. after adding succinate to the heart-muscle particles, whereas with the indirect method 6 min. elapsed between the addition of succinate and the measurement. The former is probably more relevant to reductase activity and the latter to the steady-state oxidase activity. A method which would compare the two under the same conditions is highly desirable.

In the experiments of Figs. 1 and 7 we have compared the rate of reduction of ubiquinone with the rate of formation of fumarate under very similar conditions: the particle suspensions and temperatures are identical and the times are almost the same. If one assumes agreement between the two rates, the ratio of the rates of extinction change should be equal to the ratio of the millimolar extinction coefficients.

If
$$-\frac{d[Q]}{dt} = \frac{d[fum]}{dt} \qquad (1)$$

then
$$\frac{d(\Delta E_Q/\Delta\epsilon_Q)}{dt} = \frac{d(\Delta E_{fum}/\Delta\epsilon_{fum})}{dt} \qquad (2)$$

and, since values of $\Delta\epsilon$ are not functions of time,

$$\frac{\frac{d(\Delta E_Q)}{dt}}{\frac{d(\Delta E_{fum})}{dt}} = \frac{\Delta\epsilon_Q}{\Delta\epsilon_{fum}} \qquad (3)$$

where Q represents ubiquinone, and fum represents fumarate.

Fig. 7 illustrates an experiment in which the ratio of the rates of change of extinction was 9 and the millimolar extinction coefficient of fumarate ($\Delta\epsilon_{fum}$) was found to be 0·37 cm.$^{-1}$ mM^{-1} by independent calibrations. From equation (3), $\Delta\epsilon_Q$ is 3·3 cm.$^{-1}$ mM^{-1}. If the actual value of $\Delta\epsilon$ is 10 cm.$^{-1}$ mM^{-1} then the ratio of the rate of ubiquinone reduction to the rate of fumarate production is 0·33 as compared with the value 0·36 given above. Similarly, if $\Delta\epsilon$ is 6 cm.$^{-1}$ mM^{-1} the ratio is 0·66 and a value for $\Delta\epsilon_Q$ of 3·3 would be required to give equal rates of ubiquinone reduction and fumarate production.

If participation in electron transfer requires an initial rate of ubiquinone reduction at least as great as the steady-state rate of fumarate production, these results indicate that ubiquinone is not in the main electron-transport pathway.

So far we have tacitly assumed that, as a basis for comparison of the oxidase and reductase activities of ubiquinone with the steady-state rate of oxygen utilization, the initial rate of reduction of ubiquinone must be at least equal to the steady-state rate of reduction, i.e.

$$\frac{d[QH_2]}{dt} = -\frac{1}{2}\frac{d[O_2]}{dt} = \frac{d[fum]}{dt} \qquad (4)$$

where QH_2 is reduced ubiquinone. This is appropriate only if the measured rates of change of ubiquinone are unaffected by 'back' reactions. For example, in the reduction of ubiquinone by flavoprotein,

$$\frac{d[QH_2]}{dt} = k_1[Q]\,[rfp] \qquad (5)$$

where rfp represents reduced flavoprotein, a 'back' reaction would be provided by the reoxidation of reduced ubiquinone by the adjacent oxidized component, especially since this component is fully oxidized at the time of addition of succinate:

$$\frac{d[QH_2]}{dt} = k_1[Q]\,[rfp] - k_2[QH_2]\,[oxidant]. \qquad (6)$$

Thus the maximal rate of reduction of ubiquinone may not be observed under the experimental conditions, where it is difficult to measure the reaction before a small amount of reduced ubiquinone has been formed. In order to keep the amount of oxidant as low as possible, the kinetics of reduction have usually been measured in the presence of cyanide. However, adjacent members of the respiratory chain on the oxygen side of ubiquinone may act as oxidants. Their concentration is reduced by the use of antimycin A as an inhibitor, but no measurable change of rate is observed (Fig. 5). This result suggests either that (i) the 'back' reaction does not significantly affect the kinetics or (ii) antimycin treatment does not completely eliminate the store of oxidizing equivalents. The fact that cytochrome *b* is so much more rapidly reduced in the antimycin-treated material (Chance, 1958) suggests that it can act as an oxidant of reduced ubiquinone under these conditions. Thus, as the reduction of ubiquinone is still opposed by a 'back' reaction in the presence of antimycin A, alternative (ii) appears to be the more acceptable. However, the fact that the stoicheiometric ratio of ubiquinone to cytochrome *b* is <4 weakens this argument: insufficient cytochrome *b* is present in the respiratory chain to oppose the reduction of ubiquinone during the entire course of the reaction. Thus the reduction kinetics in the presence of antimycin A would largely represent the unopposed reduction of ubiquinone and this favours the first alternative. This conclusion is supported by Fig. 5, which shows that the reduction kinetics of ubiquinone are zero order to the same extent in the presence of antimycin A as in the presence of cyanide.

Before the role of ubiquinone in electron transport can be elucidated, several questions raised by these kinetic studies must be answered. First, it will be essential to find an explanation of the difference between the maximum rate at which electron transport can occur in the steady state and the maximum rate at which electrons are transferred to ubiquinone. Secondly, the possibility that an immeasurably small amount of ubiquinone is reduced as rapidly as succinate is oxidized must be considered. Thirdly, it will be necessary to explain the zero-order kinetics of reduction of ubiquinone over such a large part of the reaction. Experiments bearing upon this problem have been published elsewhere (Chance, 1960). Finally, for a complete analysis, quantitative comparisons between ubiquinone and the other components of the respiratory chain, in terms of oxidation and reduction kinetics and of steady-state levels, must be obtained.

This paper, while extensive in its terms of reference, covers only a few of the many possible mechanisms of electron transfer and other mechanisms could make different demands upon the ubiquinone kinetics.

SUMMARY

1. An ultraviolet-extinction change is caused by addition of cyanide or antimycin A to a succinic-oxidase system. The magnitude of the change is proportional to the rate of production of fumarate.

2. The spectrum representing the extinction change caused by addition of cyanide to the succinic-oxidase system has a peak near 275 mμ and a shape resembling the difference spectrum of ethanolic solutions of reduced and oxidized ubiquinone derived from heart-muscle particles. The

former spectrum is, however, shifted to longer wavelengths.

3. The ultraviolet-extinction change is identified with the reduction of endogenous ubiquinone. No spectroscopic evidence for a semiquinone, free radical or otherwise altered reduction product has been obtained.

4. The extinction change is incomplete in the presence of succinate alone, but goes rapidly to completion in the presence of cyanide or antimycin A. About 0·5 mM-succinate gives half-maximal reduction of ubiquinone in the cyanide-inhibited system.

5. Shortly after the addition of succinate to the succinic-oxidase system, the reduction of ubiquinone is about 30% of the total reduction obtained in the presence of cyanide. This represents a steady-state reduction of ubiquinone that is less than that observed by the chemical extraction method and at longer times after adding succinate.

6. No spectroscopically detectable ubiquinone is found on the oxygen side of the antimycin-sensitive point, and insufficient ubiquinone is available to act as an 'intercytochrome carrier'.

7. Upon adding succinate to cyanide- or antimycin-treated particles, quinone is reduced in what appears to be a zero-order reaction for the first two-thirds of the reaction. The high activity of the heart-muscle preparations precluded accurate ubiquinone-reductase activity measurements by manual mixing at 26° and studies in the temperature range 7–9·5° are reported.

8. The kinetics of reduction of endogenous ubiquinone (derived from the direct spectrophotometric data) agree to within 20% with those obtained by chemical assay on light-petroleum extracts of ubiquinone. The direct data are computed on the basis of $\Delta\epsilon_{275-300\,m\mu} = 10\ cm.^{-1}\ mM^{-1}$.

9. The direct spectrophotometric and indirect chemical methods give ubiquinone-reductase rates at 7–9·5° that are about one-third of the succinic-oxidase rates.

10. A comparison of the reduction of ubiquinone and the production of fumarate made by the spectrophotometric method at 8° shows that the maximum rate of ubiquinone reduction is one-third that of the steady-state rate of fumarate production. It follows that only if the value of $\Delta\epsilon_{275-300\,m\mu}$ for ubiquinone were $3{\cdot}3\ cm.^{-1}\ mM^{-1}$ would the rates of ubiquinone reduction and fumarate production be equal.

11. The effect of the 'back' oxidation reaction upon ubiquinone-reductase activity is shown to be insignificant.

12. On the basis of the chemical mechanism proposed here the kinetic discrepancies between ubiquinone-reductase activity and succinic-oxidase activity are irreconcilable, and so far no part of the total ubiquinone has been found to be reduced at a rate equal to that at which succinate is oxidized in the steady state.

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Effects of L-Phenylalanine and Sodium Phenylpyruvate on the Formation of Adrenaline from L-Tyrosine in Adrenal Medulla *in vitro*

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While investigating the metabolism of L-tyrosine in rat-liver slices, Bickis, Kennedy & Quastel (1957) found that both L-phenylalanine and sodium phenylpyruvate inhibit the breakdown of this amino acid to acetoacetate and its incorporation into liver proteins. They suggested that these aromatic acids may affect other aspects of L-tyrosine metabolism, as, for example, the formation of hormones such as adrenaline, noradrenaline or thyroxine.

The biosynthetic pathway in bovine adrenal medulla has been established as: L-tyrosine → L-3:4-dihydroxyphenylalanine (dopa) → 3-hydroxytyramine → noradrenaline → adrenaline (Goodall & Kirshner, 1957). It is known that phenylpyruvate inhibits L-dopa decarboxylase in extracts of pig-kidney (Hartman, Akawie & Clark, 1955) and of guinea-pig adrenal medulla (Fellman, 1956), L-phenylalanine, however, having no effect. It has been suggested (Fellman, 1956) that interference with adrenaline formation by phenylalanine metabolites occurs by inhibition of L-dopa decarboxylase in adrenal medulla.

One of the phenomena associated with the inherited metabolic disorder, phenylketonuria, is a decreased concentration of plasma adrenaline. Testing 13 phenylketonuria patients and 117 normal humans, Weil-Malherbe (1955) found average basal adrenaline values of 0·62 and 1·52 μg./l. respectively. Bickel, Gerrard & Hickmans (1954) reported that the blood concentration of tyrosine in phenylketonuria patients is normal or only slightly lower, but L-phenylalanine accumulates in the blood, reaching concentrations as high as 41 mg./100 ml. (2·5 mM) (Jervis, Block, Bolling & Kanze, 1940). This value is relatively constant in individual patients even after the ingestion of phenylalanine (Borek, Brecher, Jervis & Waelsch, 1950). Phenylpyruvic acid also accumulates in the blood; the concentration in fasting patients is usually not more than 1·8 mg./100 ml. (0·1 mM), but it may increase to four times this value after the ingestion of phenylalanine (Jervis, 1952). In normal human blood, the concentration of both L-tyrosine and L-phenylalanine is approx. 10 mg./100 ml. (0·06 mM) (Johnson & Bergeim, 1951) and phenylpyruvate is not detectable.

The present paper is concerned with the formation of adrenaline by adrenal medulla *in vitro* and the effects thereon of L-phenylalanine and phenylpyruvate under conditions approximating to the physiological. Although adrenal medulla contains ascorbic acid, additional quantities of this compound were added to the media in which the tissue was incubated in order to promote the conversion of tyrosine into dopa, considered by Holtz (1959) to be the rate-limiting reaction in the synthesis of catecholamines.

EXPERIMENTAL

Materials. Adult guinea pigs (800–1200 g.) were obtained locally. DL-[2-^{14}C]Tyrosine (0·2 mC; 8·54 mg.) came from Atomic Energy of Canada Ltd. DL-[^{14}C]Adrenaline D-bitartrate (0·1 mC; 30·3 mg.) was obtained from Tracerlab Inc. L-Tyrosine, L-phenylalanine, sodium phenylpyruvate, DL-dopa, 3-hydroxytyramine–HCl, DL-noradrenaline and adrenaline (U.S.P.) were obtained from Nutritional Biochemicals Corp.

Preparation of biological material. Two guinea pigs were killed by decapitation and the adrenals were removed to chilled Ringer solution of the following composition: 0·14 M-NaCl, 6 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgSO_4$ and 1 mM-KH_2PO_4. The medullary tissue was prepared by the method of Hokin, Benfey & Hokin (1958). The bulk of the

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cortex (yellow) was removed by taking one slice from each side of an adrenal with a Stadie & Riggs (1944) slicer. The two slices were discarded and the remaining frill of cortex around the medulla (red) was trimmed off with scissors. The isolated medulla was sliced and the slices were divided evenly between two chilled Warburg vessels: a control and a test. Each vessel contained an average wet weight of 150 mg. of tissue.

Incubation technique. The reaction mixture consisted of Ringer solution, 0·01 M-phosphate buffer, pH 7·4 [0·01 M-Na_2HPO_4–0·01 M-KH_2PO_4 (81:19, v/v)], 1 mM-sodium ascorbate, 5 mM-glucose and 0·05 mM-L-[2-^{14}C]tyrosine in a total volume of 3 ml. The radioactive tyrosine was prepared by diluting DL-[2-^{14}C]tyrosine with non-radioactive L-tyrosine so that the final solution contained 81% of the L-form. The initial radioactivity of L-[2-^{14}C]tyrosine in each vessel was 43 000 counts/min. The total amount was 0·15 μmole and the specific activity was 287 000 counts/min./μmole. The tyrosine was in the side arm and the other constituents were in the main compartment. The centre well contained a paper soaked in KOH to absorb CO_2. In addition, the test vessel contained sodium phenylpyruvate or L-phenylalanine at concentrations ranging from 0·05 to 2·5 mM. The vessels were gassed with O_2 and equilibrated in a water bath at 38°. At zero time the tyrosine was tipped in and the vessels were incubated for 150 min.

Extraction of catechols. After incubation, the vessels were removed quickly to a tray of crushed ice and the reaction was stopped by the addition of 0·2 ml. of 2 N-HCl. Throughout the following procedures care was taken to keep the mixtures either cold and acidic or under N_2, in order to protect the catechols against auto-oxidation. Extractions were performed with acidic ethanol (0·1 ml. of conc. HCl/100 ml. of 95% ethanol). The medium was decanted into chilled 15 ml. plastic centrifuge tubes, and the vessels were washed with 3 ml. of acidic ethanol, the wash being added to the medium.

A stock solution of 'carrier' catechols (adrenaline U.S.P., DL-noradrenaline, 3-hydroxytyramine–HCl and DL-dopa) had been previously prepared, containing 20 μg. of each catechol/20 μl. of 0·01 N-HCl; 20 μl. of this solution was added to the medium. Thus any radioactive catechols that might have been formed were further protected against degradation by the addition of non-radioactive 'carriers'.

The medium was centrifuged at 5000*g* for 20 min. at 4°. The precipitate was washed with 3 ml. of acidic ethanol and spun in the same way for 10 min., and the combined supernatants were evaporated to dryness at low pressure (water pump) under N_2 at 30–35°. The evaporation took just over 1 hr. The residue was taken up in three portions of 0·5 ml. of acidic ethanol, transferred to a 12 ml. glass centrifuge tube, stoppered tightly and stored in the deep freeze overnight.

On the following day, the ethanol was evaporated to 0·1 ml. under a stream of N_2, the contents of the tube were centrifuged at 4°, and the supernatant was applied from a micropipette as successive 5 μl. amounts to the origin of a paper chromatogram. Between applications, the spots were dried in a stream of N_2. The residue was washed three times with 0·1 ml. of acidic ethanol and centrifuged, and the supernatant was spotted on the origin as before.

Separation of catechols. Two-dimensional paper chromatography on a sheet of Whatman no. 1 filter paper, 17 in. × 17 in. was carried out by the ascending method, the solvent systems being butan-1-ol–N-HCl–water (4:1:5, by vol.) (Hamberg & Euler, 1950) and phenol–0·1 N-HCl (85:15, w/v) (Vogt, 1952). 'Carrier' catechols (20 μl. of the stock solution) and the test solution were applied to the origin in the manner already described. The chromatogram was allowed to develop in each solvent in turn at room temperature for 36–40 hr. in an atmosphere of N_2.

Identification of catechols. The dried chromatogram was dipped in 0·44% $K_3Fe(CN)_6$ in 0·1 M-phosphate buffer, pH 7·8 (James, 1948). The catechols were immediately oxidized and appeared as coloured spots on a yellow background. The paper was dried and dipped in 0·4% ninhydrin in water-saturated butan-1-ol to locate the tyrosine spot. The test compounds were identified by their R_F values in two solvent systems and by their colours (Table 1). A total of 40 μg. of each 'carrier' catechol had been processed: 20 μg. added immediately after incubation and 20 μg. applied to the origin of the chromatogram. The ensuing spots thus gave easy identification and the method provided effective separation, as well as protection of newly synthesized radioactive catechols against loss.

It is known that the R_F values of substances may be affected by the presence of other compounds. However, in all experiments the R_F values were found to be the same as those of control compounds applied to a chromatogram as solutions in 0·1 N-HCl. This finding is in agreement with that of Euler & Hökfelt (1953), who found that there was no displacement of catecholamine spots if extractions were carried out with acidic ethanol.

Measurements of catechols. The radioactivity of the isolated spots was measured with an end-window Geiger–Müller tube (Tracerlab) which had 6% efficiency. Corrections were made for background and for absorption on the paper, and the results were finally expressed as counts/min./150 mg. wet wt. of adrenal medulla. The amount of each newly synthesized radioactive catechol was calculated from the specific activity of the experimental L-[2-^{14}C]-tyrosine and expressed as μm-moles.

Table 1. *Identification of catechols and tyrosine by paper chromatography*

For details see Experimental section.

	R_F		Colour	
	Butanol–HCl–water	Phenol–HCl	$K_3Fe(CN)_6$	Ninhydrin
Adrenaline	0·17	0·61	Pink	Violet
Noradrenaline	0·11	0·30	Pink	Brown
3-Hydroxytyramine	0·21	0·53	Blue	Brown
Dopa	0·18	0·28	Blue	Dark blue
Tyrosine	0·51	0·60	—	Purple

Table 2. *Effects of sodium phenylpyruvate and* L*-phenylalanine on the synthesis of radioactive catechols from* L*-[2-^{14}C]tyrosine in the presence of slices of guinea-pig adrenal medulla*

The incubation mixture contained 0·05 mM-L-[2-^{14}C]tyrosine, 1 mM-sodium ascorbate, 5 mM-glucose, 0·01 M-phosphate buffer, pH 7·4 (see text), Ringer solution and various concentrations of sodium phenylpyruvate and L-phenylalanine. Total volume, 3 ml.; average wet wt. of adrenal medulla, 150 mg.; incubation time, 150 min.; atmosphere, O_2; temp., 38°. Catechols were extracted from the medium with acidic ethanol and separated by two-dimensional paper chromatography. Recoveries were calculated from radioactivity of isolated spots and from specific activity of [2-^{14}C]tyrosine and expressed as μm-moles.

Sodium phenyl-pyruvate (mM)	L-Phenyl-alanine (mM)	No. of obser-vations	Dopa (μm-moles)		3-Hydroxytyramine (μm-moles)		Adrenaline (μm-moles)		Inhibition of adrenaline synthesis (%)
			Mean	Range	Mean	Range	Mean	Range	
—	—	16	0	—	7·5	3·6–12·5	2·1	1·5–2·5	—
0·05	—	3	1·3	0·5–1·8	9·4	2·7–13·6	1·3	1·1–1·6	33
0·10	—	5	2·1	0·9–3·2	6·6	3·0–12·4	1·0	0·7–1·1	50
0·25	—	3	2·3	1·6–3·2	4·6	1·7–8·4	0·2	0·04–0·4	90
0·50	—	1	2·6	—	3·2	—	0·1	—	97
—	0·10	1	0	—	5·7	—	2·3	—	—
—	0·25	1	0	—	7·1	—	1·8	—	—
—	0·50	1	0	—	9·1	—	2·0	—	—
—	2·5	1	0	—	9·5	—	2·3	—	—

RESULTS

Recovery of adrenaline

To test for loss of adrenaline during the foregoing experimental procedures, 1 μg. of DL-[^{14}C]adrenaline D-bitartrate (2·5 μm-moles of DL-[^{14}C]adrenaline) was used in place of L-[^{14}C]tyrosine. Incubation with adrenal-medulla slices, extraction, identification by paper chromatography and measurement of radioactivity were performed as before. The recovery corresponded to 100 % of the original amount of [^{14}C]adrenaline, indicating that there had been no loss during either incubation or isolation.

Synthesis of catechols from L*-[2-^{14}C]tyrosine*

Controls. The average amount of [^{14}C]adrenaline formed in 16 control flasks was 2·1 μm-moles (Table 2).

Radioactive 3-hydroxytyramine was also found. The average amount recovered was 7·5 μm-moles. However, the results showed a much wider range than with adrenaline, indicating that there might have been considerable losses. Neither [^{14}C]noradrenaline nor [^{14}C]dopa could be recovered from control flasks.

Effect of sodium phenylpyruvate. There was a marked inhibitory effect on the formation of [^{14}C]adrenaline. Phenylpyruvate concentrations of 0·05, 0·10, 0·25 and 0·50 mM caused inhibitions of 33, 50, 90 and 97 % respectively.

The effect on 3-hydroxy[^{14}C]tyramine was not as clear. The conflicting results indicate that this compound was less stable than adrenaline under our experimental conditions. No [^{14}C]noradrenaline could be detected. However, [^{14}C]dopa was recovered from all the test flasks, although there was no well-defined effect of various concentrations of phenylpyruvate.

The recovery of labelled dopa suggested that phenylpyruvate had inhibited L-dopa decarboxylase, thereby causing both an accumulation of dopa and a decreased synthesis of adrenaline.

However, there remained the possibility that the reduced recovery of [^{14}C]adrenaline might have been partly due to its degradation after synthesis. To test this possibility, 2·5 μm-moles of DL-[^{14}C]-adrenaline were incubated with adrenal-medulla slices, both in the presence and in the absence of 0·50 mM-sodium phenylpyruvate. After extraction, identification and estimation of radioactivity, the recoveries of [^{14}C]adrenaline from the control and test samples amounted to 94 and 105 % respectively. This result eliminated the possibility that phenylpyruvate had any effect on adrenaline after the latter's synthesis from L-tyrosine.

L-Phenylalanine. Addition of this substance had no effect on the recovery of radioactive adrenaline or 3-hydroxytyramine even when the concentration of L-phenylalanine was as high as 2·5 mM. As with the controls, radioactive dopa could not be detected.

DISCUSSION

Synthesis of catecholamines from L*-tyrosine*

In the present study, L-[2-^{14}C]tyrosine was converted into labelled catecholamines in the presence of slices of guinea-pig adrenal medulla. Under experimental conditions, the rate of adrenaline synthesis was 2·1 μm-moles/150 mg. wet wt. of adrenal medulla/150 min. A low rate of adrenaline synthesis from [^{14}C]tyrosine was also found by

Goodall & Kirshner (1957), who used ox-adrenal-medulla slices, and by Rosenfeld, Leeper & Udenfriend (1958), who used isolated, perfused calf adrenals.

No [^{14}C]noradrenaline could be detected. This observation was in accord with that of Hökfelt (1951), who found virtually no noradrenaline in the adrenal glands of adult guinea pigs, and with that of Hokin *et al.* (1958), who found that adrenal-medulla slices released adrenaline but no noradrenaline into the medium during an incubation period of 2 hr. These observations suggest that the *N*-methylation of noradrenaline is very efficient in the adrenal medulla of adult guinea pigs. The present results indicate that this reaction is much faster than the conversion of 3-hydroxytyramine into noradrenaline.

However, 3-hydroxy[^{14}C]tyramine was recovered. Its rate of production from L-[2-^{14}C]tyrosine was calculated to be 7·5 μm-moles/150 mg. wet wt. of adrenal medulla/150 min. This rate was 3·5 times that of adrenaline. However, the presence of monoamine oxidase in adrenal medulla (Langemann, 1951) might have caused a decrease in the yield of 3-hydroxytyramine. Pellerin & D'Iorio (1957) found equal amounts of 3:4-dihydroxyphenylacetic acid and 3-hydroxytyramine after the incubation of ox-adrenal-medulla homogenate with radioactive dopa. Goodall & Kirshner (1957) also reported a poor recovery of 3-hydroxytyramine after the incubation of ox-adrenal-medulla slices with radioactive tyrosine.

No [^{14}C]dopa could be recovered from control flasks. This finding is consistent with that of Demis, Blaschko & Welch (1955, 1956), who observed 94% decarboxylation of L-dopa by ox-adrenal-medulla homogenate during an incubation period of 2 hr. Kirshner (1959) reported that this reaction is apparently the fastest of the entire sequence in the conversion of L-tyrosine into adrenaline. Moreover, dopa is rapidly oxidized at a pH above 7. Thus it would be very unstable during an incubation period of 150 min. at pH 7·4.

Stability of adrenaline during incubation

The average amount of [^{14}C]adrenaline recovered was 2·1 μm-moles. When a similar amount of DL-[^{14}C]adrenaline (2·5 μm-moles) was incubated with adrenal-medulla slices, there was 100% recovery. Other workers have also reported on the stability of adrenaline under similar conditions. Euler & Floding (1958) incubated adrenaline with ox-adrenal-medulla homogenate for 2 hr. and observed its 'remarkable stability'. Hokin *et al.* (1958) incubated 20 μg. of adrenaline bitartrate with slices of guinea-pig adrenal medulla for 90 min. and also found that there had been no loss.

In the present experiments, adrenaline may have been protected against chemical oxidation by the presence of ascorbic acid in the medium. Heard & Welch (1935), Blaschko, Richter & Schlossmann (1937) and Euler (1956) are among those who have observed that ascorbic acid inhibits the auto-oxidation of adrenaline. Moreover, 3-hydroxytyramine may have protected adrenaline against deamination by monoamine oxidase, as suggested by Goldstein, Friedhoff & Simmons (1959). Adrenaline is a poor substrate for this enzyme, whereas 3-hydroxytyramine is an excellent one (Blaschko, 1952).

Effect of phenylpyruvate on adrenaline formation

The concentrations of aromatic acids used in the present experiments were those found in phenylketonuric blood. L-Phenylalanine, at concentrations as high as 2·5 mM, had no effect on the conversion of L-tyrosine into adrenaline by slices of guinea-pig adrenal medulla. However, 0·1 mM-sodium phenylpyruvate caused 50% inhibition of adrenaline biosynthesis. This result may be compared with that of Weil-Malherbe (1955), who found 62% reduction of plasma adrenaline in phenylketonuria.

Moreover, decrease of adrenaline synthesis in the present experiments was accompanied by an accumulation of dopa, suggesting that the decarboxylation of L-dopa was inhibited. Sodium phenylpyruvate inhibits this reaction, but L-phenylalanine has no effect. Thus, if observations made with slices of guinea-pig adrenal medulla *in vitro* can be applied to phenylketonuria, a possible explanation is found for the low plasma-adrenaline concentrations of these patients. It would seem that the accumulation of blood phenylpyruvate causes a decrease in adrenaline formation by inhibiting L-dopa decarboxylase on the biosynthetic pathway from L-tyrosine to adrenaline in the adrenal medulla.

Recent evidence indicates that dopa decarboxylase and 5-hydroxytryptophan decarboxylase are apparently the same enzyme (Westermann, Balzer & Knell, 1958; Holtz, 1959; Yuwiler, Geller & Eiduson, 1959; Werle & Aures, 1959; Fellman, 1959; Hagen, Weiner, Ono & Lee, 1960; Udenfriend, Lovenberg & Weissbach, 1960). The product of 5-hydroxytryptophan decarboxylation is serotonin, which occurs in mammalian brain and blood. Not only have low blood concentrations of serotonin been reported in phenylketonuria (Pare, Sandler & Stacey, 1957, 1958), but phenylpyruvate inhibits 5-hydroxytryptophan decarboxylase *in vitro* (Davison & Sandler, 1958). The possibility that phenylpyruvate inhibits the formation of cerebral serotonin and catecholamines might therefore be accountable in some measure for the mental abnormalities associated with phenylketonuria.

SUMMARY

1. Incubation of slices of guinea-pig adrenal medulla with L-[2-^{14}C]tyrosine in well-oxygenated modified Ringer solution results in the formation of labelled 3-hydroxytyramine and adrenaline.

2. The addition of 0·1 mM-sodium phenylpyruvate *in vitro* causes a 50% decrease in the recovery of labelled adrenaline. This decrease is caused by inhibition of the synthesis of adrenaline, rather than by its degradation after synthesis.

3. Whereas, usually, no labelled dihydroxyphenylalanine (dopa) is observed during the incubation of slices of guinea-pig adrenal medulla in the presence of labelled tyrosine, the addition of sodium phenylpyruvate results in the appearance of labelled dopa. This effect indicates that dopa decarboxylation is inhibited by sodium phenylpyruvate. It is concluded that inhibition of adrenaline biosynthesis by sodium phenylpyruvate is largely due to inhibition of dopa decarboxylase.

4. The addition of L-phenylalanine *in vitro* has no effect on the synthesis of adrenaline.

5. The bearing of these results on the mental abnormalities associated with phenylketonuria are discussed.

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Biochemistry of Dystrophic Muscle

MITOCHONDRIAL SUCCINATE–TETRAZOLIUM REDUCTASE AND ADENOSINE TRIPHOSPHATASE

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Little is known about biochemical abnormalities which accompany or are responsible for the morphological changes which occur in the primary myopathies. A number of such diseases have been characterized in humans (Walton & Nattrass, 1954). The discovery (Michelson, Russell & Harman, 1955) of a muscular dystrophy in a strain of mice, inherited by an autosomal recessive gene, has facilitated the search for biochemical alterations in myopathy. Although this condition may not be identical with any of the types of human muscular dystrophy, its investigation may help to throw light on the biochemistry of human muscular dystrophies. It seems probable that many, at least, of the secondary biochemical changes may be common to various types of muscle disease.

A few workers (e.g. Weinstock, Epstein & Milhorat, 1958; Hazzard & Leonard, 1959; White, 1959) have reported altered concentrations of certain muscle enzymes in the mouse myopathy. In view of reported morphological abnormalities in mitochondria of dystrophic mouse muscle (Dr G. W. Pearce, unpublished work; Ross, Pappas & Harman, 1960), the activity of two mitochondrial enzyme systems, succinate dehydrogenase (measured by reduction of a tetrazolium salt) and adenosine triphosphatase, was measured in dystrophic and normal mouse muscle. Observations are also recorded on the measurement and properties of these enzyme systems in normal muscle and on methods of preparing mitochondria from skeletal muscle.

MATERIALS

Abbreviation. INT, 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

Mice. These were of the inbred strain 129, bred from animals purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, U.S.A., and used when 2–3 months old. The 'normal' animals include an unknown number of mice heterozygous with respect to the recessive gene responsible for the myopathy.

Chemicals. ATP was obtained as the cryst. disodium salt from C. F. Boehringer und Soehne GmbH., Mannheim, Germany; it was neutralized with KOH before use. The cryst. barium silver salt of phosphoenolpyruvic acid, from the same source, was converted into the free acid with HCl, passed through a column of Amberlite IR-120 (H^+ form) and neutralized with KOH. Cryst. pyruvate kinase (ATP–phosphopyruvate transphosphorylase) was obtained also from Boehringer as a suspension in 2·1 M-$(NH_4)_2SO_4$. It was diluted with 4 vol. of water; assay by the method of Bücher & Pfleiderer (1955) showed that the diluted preparation was stable for several weeks, at least, when stored at −20°.

Phenazine methosulphate (*N*-methylphenazonium methosulphate), neotetrazolium [2:2′-(*p*-diphenylene)-bis-(3:5-diphenyl)tetrazolium chloride] and INT were purchased from L. Light and Co. Ltd. The corresponding formazan was prepared from INT by adding a solution of ascorbic acid to a solution of INT in 5% (w/v) Na_2CO_3 and washing the precipitate thoroughly with water; it was dried *in vacuo* over P_2O_5. 2:4-Dinitrophenol was recrystallized twice from water.

METHODS

Preparation of muscle mitochondria. Mice were killed by dislocation of the neck and bled from the throat. The hind legs were removed as quickly as possible into a cold (0–2°) solution of sucrose–EDTA (sodium salt) (0·25 M-sucrose containing mM-EDTA); the preparation was subsequently kept cold. Visible fat and nerve were removed and the muscle (normally 1–2 g. from a single mouse) was chopped finely with very sharp scissors. It was homogenized in about 5 vol. of sucrose–EDTA in a Potter–Elvehjem homogenizer with Perspex pestle and glass tube (clearance, 0·1 mm. approx.). The tube was enclosed in an ice–water jacket and the pestle rotated for 6 min. at a fairly slow speed. A further 20 vol. of medium was added and, after thorough mixing, the preparation was centrifuged for 5 min. at 600*g*. The sediment was mixed thoroughly with 20 vol. of medium (by a hand-operated homogenizer) and centrifuged as before. The combined supernatants were centrifuged (600*g*; 5 min.), and the supernatant was poured through glass wool and centrifuged for 15 min. (total time) at 13 400 rev./min. in the Spinco model L ultracentrifuge (rotor no. 40; 12 000*g*). The sedimented particles were resuspended in about 5 ml. of medium by a small homogenizer pestle that fitted loosely into the centrifuge tube, recentrifuged (12 000*g*; 15 min.) and resuspended in the same medium.

Measurements of succinate–INT-reductase activity (see below) on both homogenates and mitochondria indicated a recovery of about one-third of the mitochondria of the homogenate (assuming that the succinate–INT-reductase activity of the homogenate is confined to the mitochondria). The mitochondria recovered accounted for 2–3% of the total N of the homogenate.

The preparations were examined, as a routine, by phase-contrast microscopy to verify the absence of myofibrils. The quantity of mitochondria added to the tubes in the experiments represented 3–10 μg. of N.

Preparation of liver mitochondria. These were prepared from mouse liver by the usual techniques (Hogeboom, 1955); the homogenizing medium was sucrose–EDTA.

Measurement of adenosine triphosphatase activity. Mitochondria were shaken gently in conical centrifuge tubes at 30° for 20 min. (unless stated otherwise) in a medium containing (final concn.) tris–HCl (50 mM; pH 7·3 at 30°), KCl (75 mM), $MgCl_2$ (5 mM), EDTA (mM), ATP (4 mM), sucrose (50 mM). Phosphoenolpyruvate (8 mM) and pyruvate kinase [1 unit; a unit is defined as the amount which converts 1 μmole of substrate/min. when assayed by the method of Bücher & Pfleiderer (1955)] were added in some experiments, as indicated; other modifications of the medium are described in the text and Tables. The total volume was 0·25 ml.; all tubes were duplicated. After the incubation trichloroacetic acid (2·5%; 2·25 ml.) was added and inorganic phosphate was determined by the method of Fiske & Subbarow (1925); the modification described by LePage (1949) was used, scaled down to half quantities. Where atabrine or chlorpromazine was present in the incubation mixture, the mixture, after addition of trichloroacetic acid, was extracted twice with an equal volume of $CHCl_3$ to remove these compounds, which interfered with the determination of phosphate.

Similar tubes, which were not incubated, were set up as blanks.

Measurement of succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium-reductase activity. Mitochondria were shaken gently for 15 min. at 37° in the following medium (final vol. 1·0 ml.) in duplicate 12 ml. glass-stoppered tubes: potassium phosphate buffer (pH 7·4), 50 mM; INT, 0·1%; sodium succinate, 50 mM; sucrose, 25 mM. Trichloroacetic acid (1 ml. 10%) was added and the formazan was extracted with 4 ml. of ethyl acetate and its extinction measured at 490 mμ. When phenazine methosulphate was present in the incubation mixture the ethyl acetate was washed with an equal volume of water before reading.

Measurement of succinic-oxidase activity. This was measured at 37° by standard Warburg techniques with the use of small (approx. 7 ml.) flasks and trimethylpentane (sp.gr. 0·692) as the manometer fluid to increase the sensitivity. The medium (final vol. 1·0 ml.) contained: potassium phosphate buffer (pH 7·4), 50 mM; sodium succinate, 50 mM; cytochrome *c*, 20 μM; ATP, mM; $MgSO_4$, 2 mM; sucrose, 50 mM.

Determination of nitrogen. Total N of the tissue preparations was measured with a micro-Kjeldahl method (Johnson, 1941).

RESULTS

Homogenizing of muscle and preparation of mitochondria. Muscle was finely chopped with scissors and homogenized with a Potter–Elvehjem homogenizer in a number of different media and the homogenates were examined by phase-contrast microscopy. Homogenizing in 0·25M-sucrose led to a product with very different appearance from that obtained when the medium devised by Chappell & Perry (1954), for the preparation of mitochondria from pigeon-breast muscle, was used. This medium contains: tris (pH 7·4), 0·05M; KCl, 0·1M; $MgSO_4$, 5 mM; ATP, mM; EDTA, mM. The sucrose medium appeared to facilitate separation of the myofibrils without damaging them extensively; unless the homogenizing was very severe the striations were all retained. The other medium, although giving a rather larger yield of mitochondria, produced myofibrils that were more poorly separated, frequently misshapen and showed no striations. This difference was consistent and reproducible over a wide range of homogenizing speeds and clearances. When separated by centrifuging, the mitochondrial fraction of the homogenate in the tris medium contained many more irregular masses, probably remnants of broken myofibrils. In consequence, the succinate–INT-reductase activity of this mitochondrial fraction was about one-quarter less than that of the mitochondrial fraction obtained with the sucrose medium (expressed on the basis of equal N content of the fractions).

The difference between the two media could not be attributed to any single constituent of the tris medium. Inclusion of ATP (mM) and $MgSO_4$ (5 mM) in the sucrose produced a homogenate similar to that obtained with the tris medium. Sucrose with either ATP or $MgSO_4$ produced an intermediate appearance, as did the tris medium with the omission of both ATP and $MgSO_4$. Addition of EDTA (mM) to sucrose made no difference to the appearance of the homogenate. In view of its reported beneficial effect upon heart-muscle mitochondria (Slater & Cleland, 1952), EDTA was included in the medium in all subsequent experiments.

Adenosine triphosphatase

Conditions of measurement. Racker & Gatt (1959) demonstrated that the rate of formation of inorganic phosphate from ATP by liver mitochondria was increased by the inclusion in the medium of a system for rephosphorylating ATP. This was due to inhibiton of adenosine triphosphatase by adenosine diphosphate, the product of the reaction. Table 1 shows that similar results were obtained with mouse-skeletal-muscle mitochondria; the activity of the enzyme was increased approximately threefold by the addition of phosphoenolpyruvate and pyruvic kinase. The pyruvic kinase had no adenosine triphosphatase activity when tested under the same conditions. No inorganic phosphate was produced from phosphoenolpyruvate by mitochondria under the conditions of the assay.

Other experiments showed that there was no increase in the rate of splitting of ATP when its concentration was increased to 8 mM (in the presence of phosphoenolpyruvate and pyruvic kinase); a concentration of only 2 mM produced 29% less activity.

Effect of magnesium and dinitrophenol. Adenosine triphosphatase activity was greatly diminished when Mg^{2+} ions were omitted from the medium (Table 2). An effect of Mg^{2+} ions could be observed in the absence of the ATP-regenerating system; hence the known requirement of pyruvic kinase for Mg^{2+} ions could, at most, only accentuate the effect of Mg^{2+} ions in the complete system.

Table 1. *Effect of added adenosine diphosphate and of an adenosine triphosphate-regenerating system upon the adenosine triphosphatase of muscle mitochondria*

Mitochondria were incubated in a medium containing tris (pH 7·3) (50 mM), KCl (75 mM), $MgCl_2$ (5 mM), EDTA (mM), ATP (4 mM) and sucrose (50 mM). Other additions are indicated in the table. PEP, Phosphoenolpyruvate.

Expt. no.	Addition to medium	Inorganic phosphate formed (μg. of P)
1	—	18·5
	PEP (8 mM) + pyruvic kinase (1 unit)	55·5
2	—	7·3
	PEP (8 mM) + pyruvic kinase (1 unit)	20·0
	Adenosine diphosphate (8 mM)	1·8
3	PEP (8 mM) + pyruvic kinase (1 unit)	36·6
	PEP (8 mM) + pyruvic kinase (3 units)	33·2

Table 2. *Effect of Mg^{2+} ions and 2:4-dinitrophenol upon adenosine triphosphatase*

Mitochondria were incubated in a medium containing tris (pH 7·3) (50 mM), KCl (75 mM), EDTA (mM), ATP (4 mM), sucrose (50 mM). Phosphoenolpyruvate (8 mM) and pyruvic kinase (1 unit) were included except where indicated. Other additions are indicated in the table.

Expt. no.	Addition to medium	Inorganic phosphate formed (μg. of P)
1	—	6·2
	$MgCl_2$ (5 mM)	38·9
	—*	5·4
	$MgCl_2$ (5 mM)*	17·4
2	—	5·3
	$MgCl_2$ (5 mM)	26·5
	$MgCl_2$ (5 mM) + dinitrophenol (0·02 mM)	31·6
	$MgCl_2$ (5 mM) + dinitrophenol (0·1 mM)	38·2
	$MgCl_2$ (5 mM) + dinitrophenol (0·5 mM)	37·8
	Dinitrophenol (0·5 mM)	8·2
	—*	5·2
	$MgCl_2$ (5 mM)*	12·1
	Dinitrophenol (0·5 mM)*	7·1
	$MgCl_2$ (5 mM) + dinitrophenol (0·5 mM)*	16·0

* Phosphoenolpyruvate and pyruvic kinase omitted.

2:4-Dinitrophenol consistently stimulated the adenosine triphosphatase activity in the presence of Mg^{2+} ions. Table 2 shows that maximum stimulation occurred at a concentration of about 0·1 mM. Other experiments showed that the relative stimulation was not influenced by the duration of incubation (5–20 min.). The magnitude of the stimulation varied considerably between experiments but never exceeded 50%. There were no consistent differences in the degree of stimulation between mitochondria prepared in 0·25 M-sucrose, 0·25 M-sucrose–mM-EDTA, or the tris medium mentioned above. Variations in the severity of homogenizing had no reproducible effect, either.

The low adenosine triphosphatase activity obtained in the absence of Mg^{2+} ions was stimulated by dinitrophenol in three out of four experiments. The stimulated rate was, however, much less than that observed in the presence of Mg^{2+} ions; this was so even in the absence of phosphoenolpyruvate and pyruvic kinase, and hence was not due merely to the requirement of pyruvic kinase for Mg^{2+} ions. Typical results are presented in Table 2.

Effect of cations. Table 3 shows the adenosine triphosphatase activity of skeletal-muscle mitochondria in the presence of various metal ions. For these experiments the mitochondria were prepared and washed in sucrose–EDTA as usual, but were finally suspended in sucrose alone to avoid the presence of EDTA in the incubation medium. The activity of Co^{2+} and Mn^{2+} ions is comparable with that of Mg^{2+} ions; Ca^{2+} and Fe^{2+} ions are less active.

Effect of inhibitors. The effects of NaF, chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)-phenothiazine] and atabrine [3-chloro-7-methoxy-9-(1-methyl-4 diethylaminobutylamino)acridine] are recorded in Table 4. Each of these compounds has been reported to inhibit the adenosine triphosphatase of liver mitochondria (Löw, 1959) and chlorpromazine inhibits also brain adenosine triphosphatase (Bernsohn, Namajuska & Boshes, 1956). Chlorpromazine is evidently a potent inhibitor of the muscle adenosine triphosphatase; atabrine is less effective, and NaF inhibits only weakly. The inhibition by chlorpromazine or atabrine was relatively almost the same in the presence or the absence of dinitrophenol.

Other properties. Washing the mitochondria a second time with sucrose–EDTA did not alter their adenosine triphosphatase activity. The activity (in the presence of Mg^{2+} ions) showed a small (8%) increase when the mitochondria, suspended in sucrose–EDTA, were kept at 37° for 1 hr.

It has been shown by several workers (e.g. Lardy & Wellman, 1953) that the adenosine triphosphatase activity of carefully prepared liver mitochondria is stimulated several-fold by 2:4-dinitrophenol. The possibility was considered that

the smallness of the stimulation obtained in the present experiments with muscle mitochondria was due to the action upon the mitochondria of some factor in the supernatant fraction of the muscle homogenate. To test this a suspension of mouse-liver mitochondria was divided into two equal portions, one of which was centrifuged. The other portion of the suspension was added to 10 vol. of a muscle supernatant fraction, stood in ice for 1 hr., then centrifuged. The muscle supernatant was obtained by centrifuging a mouse-muscle homogenate for 1 hr. at 70 000*g*. In addition, the adenosine triphosphatase activity of some of the untreated mitochondria was measured in the presence of boiled supernatant fraction of muscle (0·07 ml.). The effect of dinitrophenol (four- to five-fold stimulation of adenosine triphosphatase) was not significantly influenced by the treatment of the mitochondria with the muscle supernatant or by the inclusion of the boiled supernatant in the medium. Treatment with the muscle supernatant caused a small increase in adenosine triphosphatase activity of the liver mitochondria; this may have been due to adsorption of soluble adenosine triphosphatase, since the muscle supernatant had adenosine triphosphatase activity (0·32 μg. of phosphate P liberated/μg. of N, measured under the usual conditions, and not influenced by dinitrophenol).

Table 3. *Effect of cations upon adenosine triphosphatase*

Mitochondria were incubated in a medium containing tris (pH 7·3) (50 mM), KCl (75 mM), ATP (4 mM) and sucrose (50 mM). Metallic salts were added as indicated.

Expt. no.	Addition to medium	Inorganic phosphate formed (μg. of P)
1	—	6·4
	$MgCl_2$ (mM)	38·3
	$MgCl_2$ (5 mM)	39·0
	$MgCl_2$ (10 mM)	33·0
	$CaCl_2$ (mM)	12·4
	$CaCl_2$ (5 mM)	16·3
	$CaCl_2$ (10 mM)	17·3
	$CoCl_2$ (mM)	36·0
	$CoCl_2$ (5 mM)	33·9
	$CoCl_2$ (10 mM)	21·0
2	—	1·6
	$MgCl_2$ (mM)	21·5
	$FeCl_3$ (mM)	5·5
	$FeCl_3$ (5 mM)	5·0
	$FeCl_3$ (10 mM)	4·3
	$MnCl_2$ (mM)	19·9
	$MnCl_2$ (5 mM)	25·5
	$MnCl_2$ (10 mM)	17·3

Table 4. *Inhibitors of adenosine triphosphatase*

Mitochondria were incubated in the medium of Table 1, with other additions as indicated. Atabrine and chlorpromazine were added as hydrochlorides.

Expt. no.	Addition to medium	Inorganic phosphate formed (μg. of P)
1	—	13·0
	NaF (mM)	13·3
	NaF (5 mM)	13·2
	NaF (25 mM)	8·2
	Atabrine (mM)	8·5
	Atabrine (5 mM)	3·2
	Atabrine (10 mM)	1·7
	Chlorpromazine (mM)	0·2
2	—	13·5
	Chlorpromazine (0·04 mM)	10·0
	Chlorpromazine (0·2 mM)	4·2
	Chlorpromazine (mM)	0·4
	2:4-Dinitrophenol (0·1 mM)	16·2
	Chlorpromazine (mM) + 2:4-dinitrophenol (0·1 mM)	0·7
	Atabrine (2 mM)	8·0
	Atabrine (2 mM) + 2:4-dinitrophenol (0·1 mM)	9·7

Dystrophic muscle. The adenosine triphosphatase activity of mitochondria (expressed on the basis of mitochondrial N) from muscle of four dystrophic mice is compared in Table 5 with that of normal mice in the absence or the presence of dinitrophenol. The mean unstimulated value for the dystrophic mice is slightly (15%) higher than the normal ($P < 0·02$). There is no significant difference in the degree of stimulation by dinitrophenol, although there was considerable variation.

Succinate–tetrazolium reductase

Shelton & Rice (1957) showed that the succinate dehydrogenase of liver homogenates reduced INT much more efficiently than neotetrazolium, although INT was less efficient than O_2 as an electron acceptor, in the presence of the complete succinate-oxidase system. In the present work with muscle mitochondria it was also found that INT was superior to neotetrazolium; on a molar basis approximately 20 times as much formazan was produced from INT under comparable conditions. Table 6 shows that, except with very small quantities of mitochondria, the formazan production was proportional to the amount of mitochondria present: moreover, it increased linearly with time. There was negligible INT reduction by the mitochondria when succinate was omitted from the medium.

In another experiment formazan production from INT was compared with the O_2 uptake of mitochondria from the same suspension. The quantity of formazan produced (during 10 min.) was 0·057 μmoles; this was calculated from a measured value of $20·1 \times 10^3$ for the molar extinction coefficient (490 mμ) of the formazan dissolved in ethyl acetate. The corresponding O_2 uptake (with

Table 5. *Mitochondrial adenosine triphosphatase from normal and dystrophic muscle*

Mitochondrial adenosine triphosphatase was measured as described in the Methods section; phosphoenolpyruvate and pyruvic kinase were included in the medium. The concentration of 2:4-dinitrophenol, where present, was 0·1 mM.

	Inorganic phosphate formed (μg. of P/μg. of mitochondrial N)			
	Normal		Dystrophic	
	Without dinitrophenol	With dinitrophenol	Without dinitrophenol	With dinitrophenol
	4·08	5·18	4·22	5·18
	3·62	4·16	4·46	5·07
	3·43	4·83	4·43	5·10
	4·07	5·33	4·32	5·00
Mean	3·80	4·88	4·36	5·09

Table 6. *Reduction of* 2-(p-*iodophenyl*)-3-(p-*nitrophenyl*)-5-*phenyltetrazolium by muscle mitochondria: variation with time and quantity of mitochondria*

Formazan production was measured as described in the Methods section, except that the time of incubation was varied as indicated.

Mitochondrial N present (μg.)	Time of incubation (min.)	*E*
1·38	15	0·058
2·76	15	0·164
5·52	15	0·342
11·04	15	0·696
22·08	15	1·378
22·08	10	0·921
22·08	5	0·450

Table 7. *Effect of phenazine methosulphate upon reduction of* 2-(p-*iodophenyl*)-3-(p-*nitrophenyl*)-5-*phenyltetrazolium*

Formazan production was measured as described in the Methods section, except that sodium succinate (50 mM) was added only where indicated. Other additions were as shown.

Addition to medium	*E*
Mitochondria added	
—	0·010
Succinate	0·630
Phenazine methosulphate (5 mM)	0·097
Succinate + phenazine methosulphate (5 mM)	1·26
Succinate + phenazine methosulphate (mM)	0·899
No mitochondria	
—	0
Phenazine methosulphate (2·5 mM)	0·076
Succinate + phenazine methosulphate (2·5 mM)	0·084
Sodium malonate (0·1 M) + phenazine methosulphate (2·5 mM)	0·452
Heated serum* (0·2 ml.) + phenazine methosulphate (2·5 mM)	0·703

* Human blood serum was diluted with an equal volume of water, heated for 5 min. in boiling water and filtered.

10 times the amount of mitochondria) was 15·1 μl. of O_2, measured during the 10 min. after a 10 min. equilibration period; the rate of O_2 uptake was constant for at least 50 min. Hence, when corrected to the same quantity of mitochondria, the O_2 uptake (μg.atoms) was approx. 2·5 times the formazan production (μmoles).

Further experiments were carried out to determine the effect of phenazine methosulphate upon the reduction of INT. Phenazine methosulphate can act as a direct acceptor of electrons in the oxidation of succinate by succinate dehydrogenase of heart muscle (Singer, 1959). It seemed possible, therefore, that this compound might increase formazan production by acting as intermediate electron carrier. Table 7 shows that this was the case. However, INT was also reduced non-enzymically in the presence of phenazine methosulphate; such reduction was rapid when malonate or heated human blood serum was also added. For this reason, phenazine methosulphate was not used in routine measurements of succinate dehydrogenase activity with INT.

Dystrophic muscle. There was no significant difference in the activity of mitochondria from normal and dystrophic muscle. The mean values (extinction/μg. of mitochondrial N) were respectively 0·060 (0·058–0·064) and 0·061 (0·055–0·071).

DISCUSSION

A number of workers have described procedures for the isolation of mitochondria from muscle tissue (e.g. Chappell & Perry, 1953, 1954; Kitiyakara & Harman, 1953; Holton, Hülsmann, Myers & Slater, 1957). Muscle presents considerable difficulties in this respect, on account of its toughness and, more important, the presence of the myofibrils. The mitochondria are arranged in rows between the myofibrils and the problem is to break up the cells and separate the myofibrils sufficiently to obtain an adequate yield of mitochondria without, at the same time, damaging the mitochondria or disrupting the myofibrils into small fragments which would contaminate the mitochondrial fraction. In my hands, with mouse skeletal muscle, the use of gentle but prolonged homogenizing of finely chopped muscle produced good separation of relatively undamaged myofibrils as judged by microscopic examination. The medium used by Chappell & Perry (1954) for preparing mitochondria from pigeon-breast muscle does not appear to be suitable for mouse skeletal muscle.

The relatively small stimulation of the adenosine triphosphatase of the muscle mitochondria by dinitrophenol is in marked contrast with the large stimulation of liver adenosine triphosphatase. A large response of liver adenosine triphosphatase

to dinitrophenol is considered by many workers to be an index of mitochondrial integrity. However, several authors (Chappell & Perry, 1953; Holton *et al.* 1957) have found that the adenosine triphosphatase of mitochondria from various types of muscle shows a relatively small response to dinitrophenol. Possibly this is simply the result of the greater difficulty in preparing undamaged muscle mitochondria. The finding of Chappell & Perry (1954) that adenosine triphosphatase of pigeon-breast-muscle mitochondria prepared in a medium containing tris, Mg^{2+} ions, ATP and EDTA showed a higher stimulation by dinitrophenol, was not paralleled in the present work with mouse skeletal muscle.

The approximate equivalence of Mg^{2+}, Co^{2+} and Mn^{2+} ions in activating the muscle adenosine triphosphatase is similar to the findings of Bronk & Kielly (1958) for liver adenosine triphosphatase. A lower activity of Ca^{2+} ions was found also by Chappell & Perry (1953) for pigeon-breast-muscle mitochondria.

The slightly higher adenosine triphosphatase activity of mitochondria from the dystrophic mouse muscle may explain a recent report (Zymaris, Saifer & Volk, 1960) that there is a lowered concentration of ATP in the dystrophic muscle. It is perhaps of interest that Michelazzi, Mor & Dianzani (1957), in contrast, found a decrease in mitochondrial adenosine triphosphatase in guinea-pig muscle after denervation. The presence of a normal level of succinate–INT-reductase activity in the mitochondria is of interest in view of the reported morphological changes in mitochondria in dystrophic muscle.

SUMMARY

1. The adenosine triphosphatase of mouse skeletal-muscle mitochondria was activated about equally by Mg^{2+}, Co^{2+} and Mn^{2+} ions; Fe^{3+} and Ca^{2+} ions were less active.

2. The enzyme was inhibited by adenosine diphosphate, chlorpromazine, atabrine and, weakly, by F^- ions.

3. Its activity was enhanced by 2:4-dinitrophenol, but never by more than about 50%.

4. INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride] was less efficient than oxygen as an acceptor of electrons from the succinate-dehydrogenase system of the mitochondria.

5. Mitochondria from muscle of mice with hereditary muscular dystrophy had, on the average, a 15% higher adenosine triphosphatase activity than normal (compared on the basis of equal mitochondrial nitrogen). The succinate–INT-reductase activity was normal.

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Regulation of Glucose Uptake by Muscle

5. EFFECTS OF ANOXIA, INSULIN, ADRENALINE AND PROLONGED STARVING ON CONCENTRATIONS OF HEXOSE PHOSPHATES IN ISOLATED RAT DIAPHRAGM AND PERFUSED ISOLATED RAT HEART*

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Membrane transport of glucose and intracellular phosphorylation of the sugar are both accelerated by anoxia in the perfused isolated rat heart (Morgan, Randle & Regen, 1959). In isolated rat diaphragm, membrane transport of glucose is accelerated by anoxia, but the effect of anoxia on glucose phosphorylation in this tissue is not known (Randle & Smith, 1958*a*, *b*). Crane & Sols (1953) have shown that muscle hexokinase is inhibited by glucose 6-phosphate and this observation suggested that anoxia might accelerate glucose phosphorylation in muscle by lowering the intracellular concentration of glucose 6-phosphate and thereby increasing the activity of hexokinase. The results of experiments recorded here appear to show that anoxia can lower the intracellular concentration of glucose 6-phosphate in heart and diaphragm, and further measurements have been made of the concentrations of fructose 6-phosphate and fructose 1:6-diphosphate in an attempt to define the enzymic step or steps which are affected by anoxia and which bring about this change in the concentration of glucose 6-phosphate.

The possibility that membrane transport of monosaccharides in muscle might be regulated by the intracellular concentration of glucose 6-phosphate has also been investigated by studying the effects of agents which alter the intracellular concentration of glucose 6-phosphate, on the membrane transport of D-xylose and D-3-*O*-methylglucose in isolated rat-diaphragm muscle. A preliminary report of these findings has been published (Newsholme & Randle, 1961).

MATERIALS

Rats. Hearts and diaphragms were obtained from male or female albino Wistar rats of 200–300 g. and 100–150 g. respectively, starved for 18–22 hr. beforehand.

Chemicals. Glucose 6-phosphate (disodium salt), fructose 6-phosphate (barium salt; converted into disodium salt by addition of an equivalent of Na_2SO_4 in solution), fructose 1:6-diphosphate (potassium salt), glucose 1-phosphate (dipotassium salt), ATP (disodium salt), AMP (sodium salt), TPN, DPN and tris were obtained from Sigma Chemical Co. (through George T. Gurr Ltd., London, S.W. 6). D-Xylose was obtained from T. Kerfoot and Co. Ltd., and D-3-*O*-methylglucose from Ayerst, McKenna and Harrison, New York. D-[^{14}C]Sorbitol was obtained from The Radiochemical Centre, Amersham, Bucks. Adrenaline was obtained from British Drug Houses Ltd. and dissolved in 20 mM-HCl to yield a stock solution of 1 mg./ml. This was prepared freshly and diluted to 20 μg./ml. with incubation medium. Heparin (gift of Evans Medical Ltd.) was dissolved in 0·9% NaCl to a concentration of 5000 units/ml. Crystalline insulin (a gift of Boots Pure Drug Co. Ltd.) was dissolved in 3·3 mM-HCl to yield a stock solution of 20 units/ml., which was diluted to 0·1 unit/ml. with perfusion or incubation medium.

Enzymes. Glucose 6-phosphate dehydrogenase, aldolase and glyceraldehyde 3-phosphate dehydrogenase were obtained from C. F. Boehringer und Soehne GmbH., through Courtin and Warner Ltd. Phosphohexoisomerase was obtained from Sigma Chemical Co.

Media. Hearts were perfused with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (1 mg./ml.). Diaphragms were incubated in a similar medium (except that the concentration of $CaCl_2$ was decreased by one-half). The concentration of [^{14}C]sorbitol if present was 0·1 μc/ml. The concentrations of glucose, xylose or 3-methylglucose are given in the Tables. The gas phase was O_2 or N_2 containing 5% (v/v) of CO_2.

PROCEDURE

Hearts. These were perfused for 15 min. through the coronary circulation at 37° with buffer which flowed continuously by gravity from a reservoir 60–70 cm. above the heart. At the end of perfusion the hearts were cut from the cannula and frozen immediately in acetone–solid CO_2, weighed, and kept frozen until analyses were made. The rats were injected intraperitoneally with heparin (2500 units) 20 min. or more before hearts were removed for perfusion.

Diaphragms. Intact diaphragms were incubated in 10–15 ml. of medium and hemidiaphragms in 3 ml. of medium for 20, 30, 45 or 60 min. at 37° by techniques described elsewhere (Randle & Smith, 1958*a*, *b*). In all experiments control and experimental groups of diaphragms were incubated at the same time since it is established that metabolic parameters of diaphragm can vary quantitatively from day to day (Manchester, Randle & Young, 1959). At the end of incubation the diaphragm muscle was blotted gently, weighed, and frozen in acetone–solid CO_2, if analyses were

* Part 4: Battaglia & Randle (1960).

to be made for hexose phosphates. For assay of glucose, xylose, [^{14}C]sorbitol or 3-methylglucose, muscle was extracted with boiling water (Battaglia & Randle, 1960).

METHODS

Muscle extracts. For assay of hexose phosphates, frozen heart or diaphragm muscle (one heart, or six to eight hemidiaphragms, or three to four diaphragms) were powdered in a percussion mortar chilled in solid CO_2. The frozen powder was extracted with 10% trichloroacetic acid at 0° (2 ml./g. of muscle approximately), and the supernatant solution separated by centrifuge and extracted three times with a tenfold excess of ether (to remove trichloroacetic acid). The ether was then removed by suction and *in vacuo*, and the extract brought to pH 7–8 (indicator paper, number 6883, Johnsons of Hendon Ltd.) with 10% KOH (referred to as trichloroacetic acid muscle extract).

Glucose 6-phosphate. This was assayed by measuring the change in extinction at 340 mμ in a Beckman spectrophotometer on the addition of 5 μg. of glucose 6-phosphate dehydrogenase to a mixture of 0·2 ml. of trichloroacetic acid muscle extract and 2·8 ml. of assay medium (0·06M-tris; 3 mM-EDTA; 0·1 mM-TPN; pH 8·0). Under these conditions the activity of hexokinase present as an impurity in some preparations of glucose 6-phosphate dehydrogenase was negligible (cf. Narahara & Ozand, 1960). The glucose 6-phosphate dehydrogenase preparations used were shown to be free of phosphohexoisomerase and phosphoglucomutase.

Fructose 6-phosphate. This was determined by difference, the glucose 6-phosphate content of trichloroacetic acid muscle extract being assayed (as above) in the presence or the absence of 50 μg. of phosphohexoisomerase.

Fructose 1:6-diphosphate. This was assayed by measuring the change in extinction at 340 mμ upon the addition of 200 μg. of glyceraldehyde 3-phosphate dehydrogenase to a mixture of 0·25 or 0·5 ml. of trichloroacetic acid muscle extract and 2·75 or 2·5 ml. of assay medium (0·025M-pyrophosphate; 3·3 mM-cysteine; 7·5 mM-sodium arsenate; 0·1 mM-DPN; pH 8·5) and 200 μg. of aldolase.

Glucose, xylose and [^{14}C]sorbitol. These were assayed in muscle extracts and incubation media by methods described by Battaglia & Randle (1960).

3-Methylglucose. This was assayed photometrically in muscle extracts and incubation media by Somogyi's modification of Nelson's method (Somogyi, 1945). The values for muscle extracts were corrected for blank reduction by assays performed on extracts from diaphragms incubated in the absence of 3-methylglucose.

Total water content. This was determined by weighing muscle before and after freeze-drying.

Calculations and expression of results

Hexose phosphate concentrations in trichloroacetic acid muscle extracts were calculated by reference to standard curves constructed with glucose 6-phosphate and fructose 1:6-diphosphate. The recovery of glucose 6-phosphate or fructose 1:6-diphosphate added to trichloroacetic acid muscle extracts was 85–100% in the assay and no corrections have been applied to individual values for possible recoveries of less than 100%. For calculating the hexose phosphate contents of muscle samples the volume of extract was taken to be the sum of the volume of trichloroacetic acid and the water content of the muscle sample (determined by weighing a sample of muscle powder before and after freeze-drying).

Hexose phosphate concentrations have been calculated as μmoles/100 g. of wet muscle. Incubation medium adherent to diaphragm was removed by blotting before freezing and weighing the muscle, and the wet weight used in calculating hexose phosphate concentrations was the observed weight. Hearts, on the other hand, were frozen without opening the chambers and removing retained incubation medium, and the water content was therefore found to be abnormally high. Further, anaerobic perfusion led to the retention of additional water in the extracellular compartment (cf. Morgan *et al.* 1959). The observed wet weights were therefore corrected to a water content of 79 ml./100 g. (the water content of cardiac muscle after aerobic perfusion; Morgan *et al.* 1959). The hexose phosphate concentrations in μmoles/100 g. may be converted in each case into apparent intracellular concentrations in μmoles/100 ml. of intracellular water by dividing by the intracellular volume in ml./g. (0·44 for heart, 0·60 for diaphragm; Morgan *et al.* 1959; Randle & Smith, 1958*b*). In order to calculate the true intracellular concentration knowledge of the distribution of hexose phosphates in intracellular water is required; this is not known. In order to avoid any implication that the measured concentration represents the true intracellular concentration we have preferred to express our results in terms of μmoles/100 g. rather than as intracellular concentrations. In interpreting these results the assumption is made that changes in the measured concentration will reflect similar changes in the true intracellular concentration.

RESULTS

Effects of starvation, anoxia and insulin on hexose phosphate concentrations in heart and diaphragm

The concentrations of hexose phosphates, in hearts perfused for 15 min. or diaphragms incubated for 45 min. under aerobic conditions, were dependent upon the period for which rats had been starved beforehand (Table 1). When hearts from rats starved for 18 hr. were perfused with medium containing insulin, the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate (in μmoles/100 g.) were 22, 4·4 and 3·9 respectively. With hearts from rats starved for 30 hr. the concentrations of glucose 6- and fructose 6-phosphate were significantly increased (by 100 and 43% respectively), whereas that of fructose 1:6-diphosphate was diminished by 54%. In the absence of insulin the concentration of glucose 6-phosphate was increased 50% when the period of starvation was prolonged from 18 to 30 hr. Likewise, in intact diaphragms incubated under aerobic conditions in the presence of insulin, the concentration of glucose 6-phosphate was increased from 28 to 39 when the period of starvation was prolonged from 18 to 30 hr. In studying effects of insulin or anoxia on hexose phosphate concentrations the period of starvation was carefully controlled and was 18–22 hr.

Table 1. *Effect of insulin* (0·1 *unit/ml.*) *and anoxia on the glucose* 6*-phosphate, fructose* 6*-phosphate and fructose* 1:6*-diphosphate contents of rat-heart muscle, perfused* in vitro

Hearts were perfused for 15 min. with medium containing glucose (1 mg./ml.). Rats were starved for 18 hr. unless otherwise indicated. The number of hearts is given in parentheses.

Experiment	Insulin	Hexose phosphate content (μmoles/100 g. of wet muscle) (Mean ± S.E.M.) Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1:6-diphosphate
Aerobic	−	24±1·3 (10)	4·6±0·26	3·2±0·35
Anaerobic	−	19±0·9 (10)	3·6±0·34	5·2±0·45
Difference		−5±1·6†	−1·0±0·43‡	2·0±0·57†
Aerobic	+	21±1·3 (19)	3·9±0·18	4·0±0·15
Anaerobic	+	14±0·5 (19)	2·7±0·15	5·0±0·21
Difference		−7±1·4*	−1·2±0·24*	1·0±0·26*
Aerobic	−	22±1·3 (10)	4·7±0·38	2·0±0·08
Aerobic	+	24±0·7 (10)	5·5±0·38	3·7±0·08
Difference		2±1·5	0·8±0·54	1·7±0·11*
Aerobic (starved for 18 hr.)	+	22±0·90 (29)	4·4±0·30	3·9±0·10
Aerobic (starved for 30 hr.)	+	45±1·7 (10)	6·3±2·0	1·8±0·02
Difference		23±0·8*	1·9±0·5*	−2·1±0·07*

* $P < 0·001$. † $P < 0·01 > 0·001$. ‡ $P < 0·05 > 0·01$. Other differences $P > 0·05$.

The effects of anoxia and insulin on hexose phosphate concentrations in the heart after 15 min. of perfusion are shown in Table 1. In hearts perfused aerobically without insulin the mean concentrations of hexose phosphates (in μmoles/100 g.) were glucose 6-phosphate 23, fructose 6-phosphate 4·6 and fructose 1:6-diphosphate 2·6. These correspond to intracellular concentrations of 0·51 mM-glucose 6-phosphate, 0·1 mM-fructose 6-phosphate and 0·056 mM-fructose 1:6-diphosphate (assuming uniform distribution throughout intracellular water; for volume of intracellular water see Morgan *et al.* 1959). Anoxia in the absence of insulin decreased the concentrations of glucose and fructose 6-phosphates by 21 and 22 % respectively and increased that of fructose 1:6-diphosphate by 63 %. Anoxia in the presence of insulin decreased the concentration of glucose 6- and fructose 6-phosphate by 33 and 30 % respectively and increased that of fructose 1:6-diphosphate by 20 %. Insulin (under aerobic conditions) had no significant effect on the concentrations of glucose 6- and fructose 6-phosphate but the concentration of fructose 1:6-diphosphate was increased 85 % by the hormone.

The effects of anoxia on hexose phosphate concentrations in the heart (in the presence of insulin) after 5, 10 and 15 min. are compared in Fig. 1. Although the absolute concentrations of hexose phosphates varied with the period of perfusion (especially during the first 5 min.) the effects of anoxia at 5, 10 and 15 min. were essentially similar.

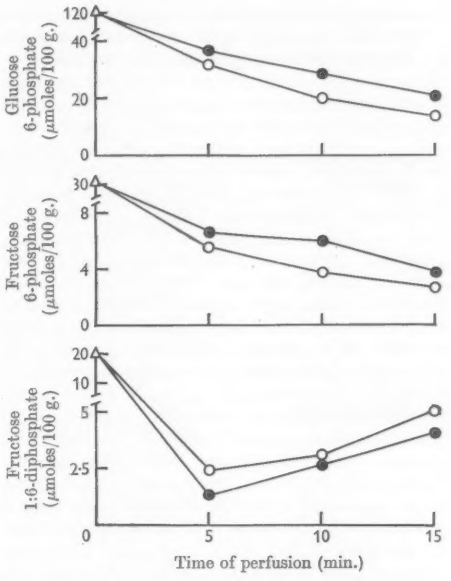

Fig. 1. Effect of anoxia on contents of hexose phosphates in hearts perfused for 5, 10 and 15 min. Hearts were perfused with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Zero-time values were obtained from hearts not subjected to perfusion. ●, Aerobic perfusion; ○, anaerobic perfusion.

The effects of anoxia, insulin and adrenaline on hexose phosphate concentrations in intact diaphragms and hemidiaphragms are shown in Table 2. In intact diaphragms incubated aerobically (but without insulin) for 20 min., the concentrations of hexose phosphates (in μmoles/100 g.) were glucose 6-phosphate 29, fructose 6-phosphate 6·7 and fructose 1:6-diphosphate 4·0. These correspond to apparent intracellular concentrations of 0·48 mM-glucose 6-phosphate, 0·11 mM-fructose 6-phosphate and 0·06 mM-fructose 1:6-diphosphate (intracellular water of 60 ml./100 g., see Randle & Smith, 1958*b*). These concentrations are very similar to those obtained in the heart (see above). Anoxia (in the absence of insulin) increased the concentrations of glucose 6-phosphate, fructose 6-phosphate, and fructose 1:6-diphosphate by 37, 16 and 33 %, whereas in the presence of the hormone anoxia increased these concentrations by 50, 86 and 65 % respectively. Insulin (under aerobic conditions) increased the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate by 50, 50 and 42 % respectively in intact diaphragms incubated for 45 min.

When hemidiaphragms were incubated aerobically, but without insulin, the mean concentrations of hexose phosphates (in μmoles/100 g.) were, glucose 6-phosphate 30, fructose 6-phosphate 7·7 and fructose 1:6-diphosphate 2·0. Anoxia in the absence of insulin was without significant effect on the concentrations of glucose 6- and fructose 6-phosphate, whereas it increased the concentration of fructose 1:6-diphosphate substantially (by 81 %). Anoxia in the presence of insulin decreased the concentrations of glucose 6- and fructose 6-phosphate by 27 and 30 % respectively and increased that of fructose 1:6-diphosphate by 33 %. Insulin under aerobic conditions increased the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate by 26, 22 and 60 % respectively.

When hemidiaphragms were incubated aerobically in the presence of insulin, adrenaline (Table 2) increased the concentrations of glucose 6- and

Table 2. *Effects of insulin* (0·1 *unit/ml.*), *adrenaline* (20 μ*g./ml.*) *and anoxia on the glucose* 6*-phosphate, fructose* 6*-phosphate and fructose* 1:6*-diphosphate contents of rat-diaphragm muscle incubated* in vitro

Intact diaphragms were incubated in medium containing glucose (2·5 mg./ml.) for 20 min. except in Expt. 3 (45 min.). Hemidiaphragms were incubated for 20 min. in medium containing glucose (5 mg./ml.). The number of muscle samples analysed is given in parentheses (see Methods).

			Hexose phosphate content (μmoles/100 g. of wet muscle) (Mean ± S.E.M.)		
Diaphragm preparation	Expt.	Insulin	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1:6-diphosphate
Intact	1. Aerobic	−	29 ± 1·9 (7)	6·7 ± 0·6	4·0 ± 0·24
	Anaerobic	−	40 ± 1·2 (7)	7·8 ± 0·6	5·4 ± 0·50
	Difference		11 ± 2·3*	1·1 ± 0·86	1·4 ± 0·55‡
	2. Aerobic	+	28 ± 2·4 (4)	6·2 ± 0·51	2·1 ± 0·08
	Anaerobic	+	43 ± 2·7 (4)	12·2 ± 0·85	3·4 ± 0·45
	Difference		15 ± 3·7†	6·0 ± 0·97†	1·3 ± 0·46‡
	3. Aerobic	−	17 ± 1·2 (11)	4·5 ± 0·5	1·5 ± 0·07
	Aerobic	+	26 ± 0·07 (11)	6·7 ± 0·45	2·1 ± 0·10
	Difference		9 ± 1·5*	2·2 ± 0·68†	0·6 ± 0·13*
Hemidiaphragm	4. Aerobic	−	28 ± 0·73 (6)	4·4 ± 0·55	2·6 ± 0·16
	Anaerobic	−	29 ± 0·73 (6)	6·0 ± 0·55	4·6 ± 0·09
	Difference		1 ± 0·98	1·6 ± 0·77	2·2 ± 0·16*
	5. Aerobic	+	49 ± 1·3 (8)	12·4 ± 0·41	2·9 ± 0·10
	Anaerobic	+	35 ± 0·49 (8)	8·5 ± 0·32	3·9 ± 0·24
	Difference		−14 ± 1·5*	−3·9 ± 0·52*	1·0 ± 0·26†
	6. Aerobic	−	33 ± 1·1 (4)	11·0 ± 1·1	1·5 ± 0·09
	Aerobic	+	41 ± 0·9 (4)	13·4 ± 1·7	2·3 ± 0·12
	Difference		8 ± 1·4†	2·4 ± 2·1	0·8 ± 0·15*
	7. Aerobic control	+	50 ± 1·3 (6)	11·9 ± 0·73	2·9 ± 0·13
	Adrenaline	+	68 ± 2·7 (6)	18·2 ± 1·1	2·6 ± 0·22
	Difference		18 ± 3·0*	6·3 ± 1·46†	−0·3 ± 0·35

* $P < 0·001$. † $P < 0·01 > 0·001$. ‡ $P < 0·05 > 0·01$. Other differences $P > 0·05$.

fructose 6-phosphate by 37 and 52% but did not increase that of fructose 1:6-diphosphate.

Effects of intracellular glucose 6-phosphate concentration and of nucleoside phosphates and hexose phosphates added in vitro *on the transport of* D-*xylose or* D-3-O-*methylglucose in the intact diaphragm*

When glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1:6-diphosphate, ATP or AMP were added *in vitro* to the incubation medium, the intracellular accumulation of xylose by diaphragm muscle was slightly increased in each instance, though many of these changes were not statistically significant. Similar effects of glucose 6-phosphate and of ATP were still evident when xylose transport was accelerated by anoxia or by fluoride respectively (Table 3) (fluoride being added to retard hydrolysis of ATP in the medium by enzymes derived from the tissue preparation).

The effects of adrenaline, fluoride and anoxia on the glucose 6-phosphate concentration and the intracellular accumulations of xylose or 3-methylglucose in intact diaphragm are given in Table 4. Adrenaline increased the glucose 6-phosphate content markedly, but had no effect on the intracellular accumulation of xylose in either the presence or the absence of insulin; on the other hand, the intracellular accumulation of 3-methylglucose was moderately increased by adrenaline. Fluoride increased glucose 6-phosphate concentration and intracellular accumulation of xylose markedly, whereas anoxia produced a slight increase in glucose 6-phosphate concentration and a moderate increase in the intracellular accumulation of xylose by diaphragm.

Table 3. *Effect of hexose phosphates and nucleoside phosphates on xylose transport in rat diaphragm* in vitro

Intact diaphragms were incubated for 30 min. in medium containing D-xylose (8·6 mM); the concentration of hexose phosphates or nucleoside phosphates was 10–15 mM. There were four observations in each group.

Addition to medium	Intracellular xylose space (ml./100 g. of wet muscle) (Mean ± S.E.M.)	Difference ± S.E. of difference
None	5±1·4	
Glucose 6-phosphate	6±0·9	1±1·7
None (anaerobic)	18±1·7	
Glucose 6-phosphate	21±1·8	3±2·4
None	14±2·2	
Glucose 1-phosphate	17±0·9	3±2·4
None	8±1	
ATP	13±0·6	5±1·2*
Fluoride (10 mM)	21±1·4	
Fluoride + ATP	26±1·6	5±2·1
None	8±2·6	
Fructose 6-phosphate	14±2	6±3·3
None	11±1·4	
Fructose 1:6-diphosphate	18±1·7	7±2·2†
None	10±2·0	
AMP	15±2·7	5±2·9

* $P < 0{\cdot}01 > 0{\cdot}001$.
† $P < 0{\cdot}02 > 0{\cdot}01$. Other differences $P > 0{\cdot}05$.

DISCUSSION

The ratio of intracellular glucose 6-phosphate to fructose 6-phosphate, was not affected by anoxia, insulin or adrenaline in either heart or diaphragm (although the ratios, which averaged 4·5:1, were consistently greater than the ratio of 2·33:1 obtained at equilibrium with phosphohexoisomerase). Because the ratio was not affected by these agents, it seems unlikely either that the phosphohexoisomerase step is rate-limiting for glycolysis under any of the experimental conditions employed or that effects of anoxia or insulin on the rate of glycolysis are to be attributed to changes in the rate of the phosphohexoisomerase reaction.

In the perfused rat heart, anoxia diminished the concentrations of glucose and fructose 6-phosphates, but increased the concentration of fructose 1:6-diphosphate (in either the presence or the absence of insulin). In hemidiaphragms, anoxia diminished concentrations of glucose 6- and fructose 6-phosphate and increased that of fructose 1:6-diphosphate, when insulin was present; in the absence of insulin, anoxia raised the concentration of fructose 1:6-diphosphate without affecting those of glucose 6- and fructose 6-phosphate. In these tissues both the rate of formation of glucose 6- and fructose 6-phosphate and the rate of flow through the sequence glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate → lactate are accelerated by anoxia in either the presence or the absence of insulin. [In the absence of insulin anoxia accelerates glucose transport and thereby glucose phosphorylation in diaphragm and heart (Randle & Smith, 1958*a*; Morgan *et al.* 1959) and glycogen breakdown (unpublished observations); in the presence of insulin, transport is not affected by anoxia, which nevertheless accelerates glucose phosphorylation in the heart, but not in the diaphragm (Morgan *et al.* 1959); anoxia accelerates glycogen breakdown in both heart and diaphragm (unpublished observations). Lactate formation is accelerated by anoxia in both tissues.] Thus the overall rate of conversion of fructose 6-phosphate into the diphosphate was accelerated to a much greater extent by anoxia than either the rate of formation of glucose 6- and fructose 6-phosphate or the conversion of the diphosphate into lactate. We conclude therefore that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate is specifically increased by anoxia and that this is an

Table 4. *Effect of adrenaline* (20 μg./ml.), *fluoride* (10 mM) *and anoxia on the glucose 6-phosphate content and transport of* D-*xylose or* D-3-O-*methylglucose in isolated rat diaphragm* in vitro

Intact diaphragms were incubated for 60 min. (Expts. 1–4) or 20 min. (Expt. 5). The concentrations were: glucose, 2·5 mg./ml.; xylose, 5 mg./ml. (Expts. 1, 3 and 4) or 3·75 mg./ml. (Expt. 5); 3-methylglucose, 5 mg./ml.; insulin, 0·1 unit/ml. The number of observations is given in parentheses. In Expts. 1, 3, 4 and 5, glucose 6-phosphate was assayed in single extracts prepared from all diaphragms incubated.

Expt.	Insulin	Glucose	Non-utilizable sugar	Glucose 6-phosphate concn. (μmoles/100 g.)	Intracellular xylose or 3 methylglucose space (ml./100 g.) (Mean ± S.E.M.)	(Mean difference ± S.E. of difference)
1. Control	–	–	Xylose	35	7±1·0 (10)	
Adrenaline	–	–		176	7±0·75 (10)	0±1·3
2. Control	–	–	3-Methylglucose	—	11±1·7 (6)	
Adrenaline	–	–		—	19±1·4 (6)	8±2·2†
3. Control	+	–	Xylose	35	19±1·0 (4)	
Adrenaline	+	–		176	24±2·8 (4)	5±3·0
4. Control	–	–	Xylose	35	2±0·8 (6)	
Fluoride	–	–		94	12±0·7 (6)	10±1·1*
5. Aerobic	–	+	Xylose	24	7±2·0 (6)	
Anaerobic	–	+		33	14±1·3 (6)	7±2·4‡

* $P < 0.001$. † $P < 0.01 > 0.001$. ‡ $P < 0.02 > 0.01$. Other differences $P > 0.05$.

important rate-limiting step for glycolysis under aerobic conditions. This effect of anoxia could result either from an increase in the rate of the phosphofructokinase reaction or from a diminution in the rate of the fructose 1:6-diphosphatase reaction. These two possibilities cannot be distinguished from the results obtained in the present study.

In intact diaphragm, on the other hand, anoxia increased the concentrations of all three hexose phosphates in either the presence or the absence of insulin. In intact diaphragm therefore anoxia accelerated glucose 6- and fructose 6-phosphate formation to a much greater extent than the overall conversion of fructose 6-phosphate into the diphosphate (though the latter was clearly accelerated to a greater extent than was the conversion of the diphosphate into lactate). The increase in diphosphate concentration observed could in this instance have been brought about by the rise in fructose 6-phosphate concentration. Thus although anoxia does accelerate the overall rate of formation of the diphosphate in intact diaphragm, it is not possible to conclude from the hexose phosphate concentrations in this tissue that the overall phosphorylation of fructose 6-phosphate was rate-limiting for glycolysis under aerobic conditions, or that anoxia accelerated glycolysis in this preparation by a specific effect on this step.

In the perfused rat heart, insulin increased the concentration of fructose 1:6-diphosphate without affecting that of glucose 6- or fructose 6-phosphate. Under similar conditions the hormone has been found to accelerate the formation of glucose 6-phosphate (by accelerating transport of glucose) and to increase the rate of flow through the sequence glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate → lactate (shown by increased lactate production; unpublished observations). This would suggest that insulin may also increase the overall rate of formation of fructose 1:6-diphosphate from fructose 6-phosphate in a specific way. Since, however, glucagon can accelerate the formation of lactate from glycogen and the phosphorylation of glucose in the perfused heart (Cornblath, Morgan & Randle, 1961; M. J. Henderson, H. E. Morgan & P. J. Randle, unpublished work), and since glucagon is present as a contaminant in many insulin preparations there is the possibility that these effects of insulin may have been due to glucagon present as a contaminant in the preparations of insulin employed rather than to insulin itself.

In either hemidiaphragms or intact diaphragms, insulin increased the concentrations of all three hexose phosphates. The hormone also increased the rate of formation of glucose 6-phosphate (by accelerating glucose transport) and the rate of lactate production from glucose in diaphragm muscle under similar conditions (unpublished observations). Thus although the hormone did accelerate the overall conversion of fructose 6-phosphate into the diphosphate, this effect could have been brought about by an increase in the fructose 6-phosphate concentration rather than by a specific effect on the phosphofructokinase or fructose 1:6-diphosphatase reactions. On the other hand adrenaline, which (like insulin) increased the concentrations of glucose 6- and fructose 6-phosphate in hemidiaphragms, did not (unlike insulin) increase the diphosphate concentration.

In hearts from rats in which the period of starvation before perfusion of the heart had been prolonged to 30 hr., glucose 6- and fructose 6-phosphate concentrations were markedly increased, whereas that of fructose 1:6-diphosphate was markedly lowered. Thus it would appear that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate may be reduced in a specific way by prolonged starving. A decreased concentration of circulating insulin as a consequence of starvation could be one important factor in this respect.

As Crane & Sols (1953) have shown, the activity of muscle hexokinase is inhibited non-competitively by glucose 6-phosphate, the K_i for heart muscle hexokinase being of the order of 0·1 mM (Sols & Crane, 1954; Crane & Sols, 1955). The intracellular concentration of glucose 6-phosphate in rat hearts perfused under aerobic conditions was 0·51 mM in the present investigation (assuming that glucose 6-phosphate is uniformly distributed throughout intracellular water and that none is combined with intracellular protein). In rat heart therefore hexokinase is likely to be subject to inhibition by glucose 6-phosphate and the accelerating effect of anoxia on phosphorylation of glucose by rat heart noted by Morgan *et al.* (1959) could result from the fall in intracellular glucose 6-phosphate concentration that this agent has been shown to induce.

Other points of evidence that support the view that the intracellular glucose 6-phosphate concentration may regulate the activity of muscle hexokinase and thereby glucose phosphorylation may be summarized as follows. In the rat, prolonged starvation increased the concentration of glucose 6-phosphate in heart and diaphragm, substantially; Kipnis (1959) has already shown that phosphorylation of glucose in diaphragm muscle is impaired by starvation, and in rat heart also glucose phosphorylation appears to be moderately depressed after 30 hr. of starvation (unpublished observations). Adrenaline, in the presence of insulin, increased the intracellular concentration of glucose 6-phosphate markedly in accordance with long-standing observations concerning effects of adrenaline on the glucose 6-phosphate content of muscle (Cori & Cori, 1931–32; Hegnauer & Cori, 1934; Sutherland, 1952); in parallel experiments in which the effect of adrenaline on glucose space in the intact diaphragm was investigated, adrenaline caused intracellular accumulation of glucose (glucose space in the absence of adrenaline $-4 \pm 0{\cdot}7$ ml./100 g.; in the presence of adrenaline $7 \pm 1{\cdot}9$). It is deduced from this change that adrenaline action reduces the rate of glucose phosphorylation in diaphragm muscle in the presence of insulin (for discussion of significance of intracellular glucose accumulation see Park *et al.* 1956). Substantial accumulation of glucose in muscle cells *in vivo* follows the administration of adrenaline to rats (Kipnis, Helmreich & Cori, 1958).

Changes in the intracellular concentration of glucose 6-phosphate in heart and diaphragm can therefore be correlated with changes in the rate of glucose phosphorylation. It seems reasonable to conclude that the intracellular concentration of glucose 6-phosphate is important in the regulation of glucose phosphorylation by hexokinase in muscle, though it should be emphasized that this may not be the only factor involved. Lynen, Hartmann, Netter & Schuegraf (1959) found that anoxia, cyanide and 2:4-dinitrophenol, which increase the rate of glucose phosphorylation in yeast, reduce the intracellular concentration of glucose and fructose 6-phosphate and increase that of fructose 1:6-diphosphate in yeast. Yeast hexokinase (unlike muscle hexokinase) is not inhibited by glucose 6-phosphate and Lynen *et al.* have proposed that in yeast, intracellular phosphorylation both of glucose and of fructose 6-phosphate may be regulated by the availability of ATP to hexokinase and phosphofructokinase. The possibility that such a mechanism may also contribute to effects of anoxia on glucose phosphorylation in muscle has neither been adduced nor refuted by the present investigation.

Although intracellular glucose 6-phosphate appears to be an important regulator of glucose phosphorylation in muscle, it seems unlikely to be an important factor in the regulation of transport of monosaccharides. Glucose 6-phosphate added to the incubation medium *in vitro* accelerated xylose transport in diaphragm to a small extent but this effect was also seen with a number of other hexose phosphates and nucleoside phosphates and had previously been found with inorganic phosphate (Randle & Smith, 1958*b*). The mechanism of this effect is not known. In some instances increases in intracellular glucose 6-phosphate (e.g. in diaphragm with anoxia or fluoride) were associated with accelerated xylose transport, but adrenaline (which produced the greatest increase in intracellular glucose 6-phosphate) had no detectable effect on xylose transport. The more rapidly transported sugar 3-methylglucose did penetrate diaphragm more rapidly in the presence of adrenaline. Moreover, anoxia, which accelerates monosaccharide transport in rat heart, causes a fall in intracellular glucose 6-phosphate in this tissue. Thus it seems likely that accelerating effects of anoxia or fluoride on monosaccharide transport in muscle are related to changes other than the intracellular concentrations of glucose 6-phosphate, e.g. to the availability of high-energy phosphate compounds to the membrane transport system for monosaccharides (Randle & Smith, 1958*a*, *b*).

The results of this and earlier studies appear to show that at least two factors are responsible for the

Pasteur effect in muscle (inhibition of glycolysis by oxygen). One factor is a diminished rate of glucose transport under aerobic conditions, and this is particularly important in the absence of insulin where glycolysis is limited by glucose-transport rate. The other factor is the rate of phosphorylation of glucose by hexokinase which is inhibited under aerobic conditions; this is particularly important in heart muscle where in the presence of insulin glycolysis is limited by the rate of phosphorylation of glucose. The results of the present study suggest that the regulation of glucose phosphorylation is indirect and that it depends upon the intracellular concentration of glucose 6-phosphate, which is regulated by the rate of conversion of fructose 6-phosphate into fructose 1:6-diphosphate.

SUMMARY

1. The concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate have been assayed in extracts of perfused rat heart and isolated rat diaphragm by enzymic methods, and effects of anoxia, insulin and adrenaline on these parameters investigated.

2. In rat hearts perfused aerobically concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate (in μmoles/100 g.) were 23, 4·6 and 2·6. The corresponding concentrations in intact diaphragms were 29, 6·7 and 4·0 and in hemidiaphragms 30, 7·7 and 2·0.

3. In rat heart these concentrations were changed (as per cent of control) as follows: anoxia -21, -22 and $+63$; insulin $+9$, $+17$ and $+85$; anoxia in the presence of insulin -33, -30 and $+20$. In intact diaphragms these concentrations were changed by anoxia $+37$, $+16$ and $+33$; by insulin $+50$, $+50$ and $+42$; by anoxia in the presence of insulin $+50$, $+86$ and $+65$. In hemidiaphragms these were changed by anoxia $+4$, $+36$ and $+81$; by insulin $+26$, $+22$ and $+60$; by anoxia in the presence of insulin -27, -30 and $+33$; adrenaline in the presence of insulin $+37$, $+52$ and -12.

4. It is concluded that in heart and diaphragm the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate may limit glycolysis under aerobic conditions and that anoxia accelerates glycolysis by increasing the rate of this step in particular.

5. Evidence is presented that phosphorylation of glucose by hexokinase may be regulated in muscle by the intracellular concentration of glucose 6-phosphate (a known inhibitor of muscle hexokinase). On the other hand, monosaccharide transport in muscle does not appear to be regulated by the intracellular concentration of glucose 6-phosphate.

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Pyridoxal Phosphate Breakdown by an Alkaline-Phosphatase Preparation

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Pogell (1958) has demonstrated the presence of phosphatase activities for pyridoxal and pyridoxamine phosphates in rabbit-liver fractions. Wada *et al.* (1959) have also demonstrated the breakdown of pyridoxal and pyridoxamine phosphates in rabbit-liver preparations. Roberts, Rothstein & Baxter (1958) have obtained results indicating the presence of pyridoxal phosphate-phosphatase activity in brain tissue, and these results have been confirmed by Begum & Bachawat (1960), who suggest that a non-specific acid phosphatase is involved. Evidence for pyridoxal phosphate destruction in *Neurospora crassa* extracts has been obtained by Wainwright (1959), and for pyridoxamine phosphate breakdown in the fungus *Ophiostoma multiannulatum* by Wikberg (1960). The latter author has suggested that an acid phosphatase is involved. Turner & Happold (1961) have demonstrated pyridoxal phosphate-phosphatase activity in *Escherichia coli* extracts. By the use of direct spectrophotometric methods for the assay of activity, they suggest that an alkaline phosphatase is involved.

The following report shows that pyridoxal phosphate and pyridoxamine phosphate are readily hydrolysed by purified alkaline-phosphatase preparations.

EXPERIMENTAL

Materials

Alkaline phosphatase. An 'Intestinal phosphatase' preparation, of bovine origin, was obtained from Armour Laboratories, Chicago, Ill., U.S.A. A similar preparation was obtained, for purposes of comparison, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chemical compounds. Pyridoxal phosphate and pyridoxamine phosphate, also pyridoxal and *p*-nitrophenyl phosphate, were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Versenol (*N*-hydroxyethylethylenediaminetriacetic acid, trisodium salt) was obtained from the Bersworth Chemical Co., Framington, Mass., U.S.A.

Assay of phosphatase activities

Pyridoxal phosphate as substrate. The standard composition of reaction mixtures was: buffer, 0·04 M-veronal, pH 8·9, 2·0 ml.; enzyme preparation, 0·1 mg. of protein, in aqueous solution, 0·1 ml.; pyridoxal phosphate, 0·4 μmole, in 0·4 ml.; water or additions to give a total volume of 4·0 ml. All reactions were carried out at 37° for 4 min. unless otherwise stated. Reactions were stopped by the addition of 0·5 ml. of 2·5 N-NaOH, and absorption values were read at 390 and 300 mμ, i.e. $\lambda_{max.}$ values for substrate and product, against the appropriate blanks. The amount of pyridoxal phosphate and the free base present were calculated as described by Turner & Happold (1961). At the pH of 0·28 N-NaOH, i.e. approx. pH 13, $\epsilon^{300}_{\text{pyridoxal}} = 7000$; $\epsilon^{390}_{\text{pyridoxal}} = 800$; $\epsilon^{300}_{\text{pyridoxal phosphate}} = 1200$; $\epsilon^{390}_{\text{pyridoxal phosphate}} = 6300$. From these measured values, it can be shown that:

$$[\text{pyridoxal phosphate}] = (1{\cdot}63\,E^{1\,\text{cm.}}_{390} - 0{\cdot}19\,E^{1\,\text{cm.}}_{300}) \times 0{\cdot}1\ \text{mM}$$
$$[\text{pyridoxal}] = (1{\cdot}48\,E^{1\,\text{cm.}}_{300} - 0{\cdot}28\,E^{1\,\text{cm.}}_{390}) \times 0{\cdot}1\ \text{mM}$$

A Cary recording spectrophotometer, model 14, was used to record spectra and to determine the above extinction coefficients. Standard solutions of pyridoxal and its phosphate were diluted to 0·05 mM with buffer and NaOH, and measurements were made in quartz cells of 1 cm. light-path. A Beckman DU spectrophotometer was used for routine absorption measurements at $\lambda_{max.}$ values.

By using controls in which water replaces substrate, the absolute amounts of pyridoxal and its phosphate also can be calculated. For example, with a standard solution containing pyridoxal phosphate and pyridoxal in 0·28 N-NaOH, where each component was 0·050 mM, $E^{1\,\text{cm.}}$ values at 300 and 390 mμ were found to be 0·405 and 0·358 respectively. By substituting these values in the above equations, the respective concentrations of pyridoxal phosphate and pyridoxal are calculated to be 0·051 and 0·050 mM.

In practice it is convenient to use controls in which substrate or enzyme is added after the NaOH. If absorption values are read at 300 mμ with the control as blank, and the control itself is read at 390 mμ with the stopped reaction mixture as blank, then the changes in both substrate and product concentrations can be calculated. A simple expression can be derived with which it is possible to calculate the mean amount of substrate utilized and product formed. Thus for a stopped reaction mixture having the composition given above:

$$\text{Mean } \Delta \text{ substrate and mean } \Delta \text{ product} = (0{\cdot}304\,.\,\Delta E^{1\,\text{cm.}}_{390} + 0{\cdot}290\,.\,\Delta E^{1\,\text{cm.}}_{300})\ \mu\text{mole.}$$

Pyridoxamine phosphate and p*-nitrophenyl phosphate as substrates.* Reaction-mixture composition was the same as when pyridoxal phosphate was substrate except that 2·0 μmoles of substrate was used. The higher substrate concentration was necessary owing to greater reaction velocities with the above-mentioned compounds. After incubation at 37°, reactions were stopped by the addition of NaOH. Phosphatase activity was measured by the assay

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of inorganic phosphate, according to the method of Gomori (1941–42). A Beckman DU spectrophotometer was used for absorption measurements at 675 mμ. In a number of experiments with *p*-nitrophenyl phosphate, this compound was directly substituted for pyridoxal phosphate in the procedure previously described but a shorter incubation time was used. The absorption of stopped reaction mixtures was read at 400 mμ, the appropriate blank being used.

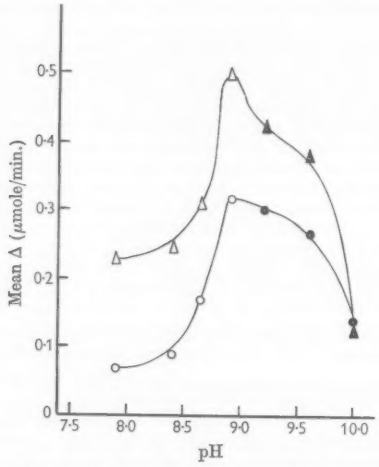

Fig. 1. Effect of pH on pyridoxal phosphate breakdown. Reaction mixtures contained: 2·0 ml. of 0·04 M-buffer; 0·1 ml. of enzyme solution (approx. 1 mg./ml.); 0·4 ml. of pyridoxal phosphate solution (containing 0·4 μmole); 1·5 ml. of water or $MgSO_4$ solution. Reactions were carried out at 37° for 4 min. and then stopped by the addition of 0·5 ml. of 2·5 N-NaOH. Absorption values were read at 300 and 390 mμ, blanks which contained water in place of substrate being used. Concentrations of pyridoxal and pyridoxal phosphate present were calculated as described in the text. These values are expressed as the mean of pyridoxal phosphate utilized and free pyridoxal formed. Upper curve, pyridoxal phosphate breakdown in the presence of 2·0 mM-$MgSO_4$. ○ and △, Results with veronal buffers; ● and ▲, results with Na_2CO_3–$NaHCO_3$ buffers.

Units of activity. Activities are expressed in terms of μmoles/mg. of protein/min. Protein concentrations were determined by a quantitative biuret procedure. In cases where the protein concentration was not accurately measured, activities are expressed as amount of change (μmole) under the conditions described.

RESULTS

pH *optimum for pyridoxal phosphate breakdown.* Preliminary experiments showed the pH optimum to be in the range 8–10. Veronal buffers (Britton & Robinson, 1931) were used in the range 8–9 and sodium carbonate–sodium hydrogen carbonate buffers (Delory & King, 1945) between pH 9 and 10. The pH optimum was found to be approx. 8·9. Results are illustrated in Fig. 1. It was concluded that ammediol (2-amino-2-methyl propane-1:3-diol) buffers (Holmes, 1943) should not be used in the range pH 8–10, owing to differences observed in the absorption spectra of both pyridoxal and pyridoxal phosphate when these are plotted at pH 10 with ammediol rather than sodium carbonate–sodium hydrogen carbonate buffer.

Effect of substrate and enzyme concentrations. With the standard reaction-mixture composition, initial rates of pyridoxal phosphate breakdown at 37° were found to be proportional to the amount of enzyme up to a value of at least 0·16 mg./4 ml. Under similar conditions, with 0·10 mg. of enzyme, substrate utilization was linear with time up to approx. 8 min., and reaction rates remained constant at substrate concentrations above approx. 0·05 mM, i.e. 0·2 μmole/4 ml. (see Table 1). At an enzyme concentration of 0·3 mg./4 ml. the variation of substrate concentration between 0·02 and 0·17 mM gave a double-reciprocal plot (Lineweaver & Burk, 1934), indicating approx. K_m 0·05 mM (see Fig. 2). With *p*-nitrophenyl phosphate as substrate, with the same conditions except for an incubation time decreased from 4 min. to 2·75 min., and a similar spectrophotometric-assay procedure, approx. K_m 0·06 mM was obtained. K_m values were calculated from visually drawn straight lines.

Table 1. *Effect of substrate concentration on pyridoxal phosphate breakdown*

Reaction mixtures had the standard composition described in the Experimental section, except that substrate concentration was varied as indicated. In each case, 0·1 mg. of enzyme was present (cf. Fig. 2), and incubation was for 4 min. at 37°.

$10^4 \times$ Initial substrate concn. (M)	$\Delta E^{1\,cm.}_{390\,m\mu}$	$\Delta E^{1\,cm.}_{300\,m\mu}$	Substrate utilized (μmole/mg. of protein/min.)	Mean Δ substrate and product (μmole/mg. of protein/min.)
0·28	0·070	0·073	0·115	0·108
0·50	0·090	0·092	0·143	0·135
0·75	0·095	0·100	0·153	0·145
1·00	0·092	0·094	0·148	0·138
1·25	0·085	0·092	0·135	0·133
2·25	0·083	0·103	0·130	0·138

Effect of magnesium ions and N-hydroxyethylethylenediaminetriacetate. Magnesium ions were found to increase phosphatase activity with pyridoxal phosphate as substrate. The optimum Mg^{2+} ion concentration was found to be approx. 2 mM (see Table 2). At this concentration, the rate of pyridoxal phosphate breakdown was increased by 56%. The pH optimum for phosphatase activity, measured in the presence of Mg^{2+} ions, was the same as that observed in their absence (see Fig. 1).

The metal-chelating agent trisodium *N*-hydroxyethylethylenediaminetriacetate inhibited pyridoxal phosphate breakdown at all concentrations used, without preincubation with the enzyme (see Table 2).

Effect of various compounds on pyridoxal phosphate-phosphatase activity. Pyridoxamine phosphate was found to behave as a competitive inhibitor of pyridoxal phosphate breakdown. With variable inhibitor concentrations and with two different substrate concentrations, the breakdown of pyridoxal phosphate was measured spectrophotometrically by the previously described method. Control experiments showed that the possible simultaneous breakdown of inhibitor did not cause any change in absorption at the wavelengths used for the assay procedure. The simple graphical method of Dixon (1953) was used to obtain the inhibition constant directly, a value of K_i 0·15 mM being found (see Fig. 3). K_m values were also obtained from Fig. 3, by virtue of the fact that the extrapolation of each linear plot cuts the base line at a value of i equal to $-K_i\left(\frac{s}{K_m}+1\right)$ (Dixon & Webb, 1958). K_m values thus derived were 0·08 and 0·06 mM. At the higher substrate

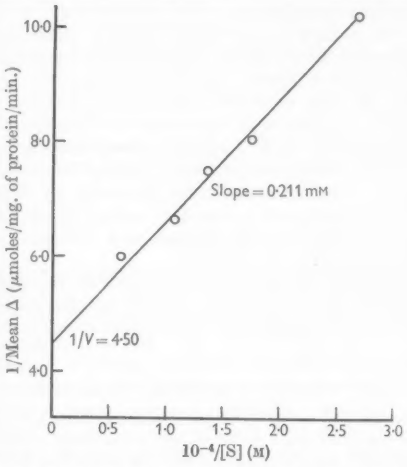

Fig. 2. Effect of substrate concentration on pyridoxal phosphate breakdown. Reaction-mixture composition was as described in Fig. 1, except that 0·3 mg. of enzyme was present, and initial substrate concentration varied as indicated. No metal ions were added. Reaction rates were calculated as described in the text.

Table 2. *Effect of magnesium ions and N-hydroxyethylethylenediaminetriacetate on the hydrolysis of pyridoxal phosphate*

Standard reaction-mixture composition and conditions were used, and results were calculated as described in the text. In the Mg^{2+} ion experiments, reaction was started by the addition of substrate, $MgSO_4$ solution and enzyme being allowed to preincubate in buffer for approx. 30 min. With *N*-hydroxyethylethylenediaminetriacetate (Versenol) reactions were started by the addition of enzyme and no preincubation of these two components occurred. The Mg^{2+} ion and Versenol experiments were carried out on separate occasions.

Addition	Concn. of addition (mM)	Mean Δ substrate and product (μmole/4 min.)	Activity (%)
$MgSO_4$	0	0·108	100
	2·0	0·144	133
	2·0	0·168	156
	0·2	0·148	137
	0·02	0·134	124
Versenol	0	0·073	100
	0·27	0·044	60
	0·47	0·035	48
	0·67	0·012	17
	2·0	0·006	8

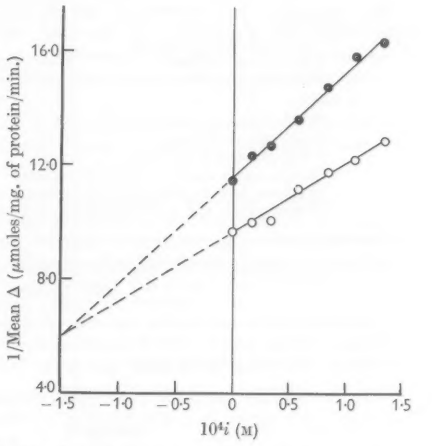

Fig. 3. Effect of pyridoxamine phosphate on pyridoxal phosphate breakdown. Reactions were carried out under the standard conditions previously described, with inhibitor concentrations shown. The two substrate concentrations used were 0·067 mM (●) and 0·133 mM (○).

concentration, an equivalent concentration of pyridoxamine phosphate caused 25% inhibition, at the lower concentration, 18% inhibition.

Under similar conditions pyridoxal had no inhibitory effect on pyridoxal phosphate breakdown when used at concentrations between 0·25-fold and 1·66-fold substrate concentration, i.e. up to 0·166 mM.

Inorganic orthophosphate inhibited pyridoxal phosphate hydrolysis competitively, the value of K_i being determined by the method of Dixon (1953) as approx. 0·06 mM. Under standard reaction conditions, with substrate at a concentration of 0·1 mM, an equivalent amount of inorganic phosphate caused 43% inhibition.

No effect of NH_4^+ ions on pyridoxal phosphate-phosphatase activity could be detected at concentrations between approx. 0·17-fold and 1·66-fold substrate concentration, i.e. 0·1 mM.

Pyridoxamine phosphate as substrate of alkaline phosphatase. It was not possible to investigate the breakdown of pyridoxamine phosphate spectrophotometrically. The spectra of the free base and its phosphate ester are similar at all pH values. Accordingly, rates of hydrolysis were measured by assay of the inorganic phosphate produced. The composition of reaction mixtures is described in the Experimental section.

Preliminary experiments showed that rapid hydrolysis of pyridoxamine phosphate was catalysed by the alkaline-phosphatase preparation, and that the pH optimum was approx. pH 8·9. A direct comparison of the rates of hydrolysis of pyridoxamine and pyridoxal phosphates showed that the former was approx. 1·5-fold that of the latter. Similar ratios were observed by Pogell (1958). These results, together with those obtained with *p*-nitrophenyl phosphate as a standard, are given as Expt. 1 in Table 3.

Specificity of phosphatase action. To determine whether the same species of enzyme was responsible for the hydrolysis of pyridoxal, pyridoxamine

Table 3. *Comparative rates of hydrolysis of pyridoxal, pyridoxamine and* p-*nitrophenyl phosphates*

In Expt. 1, the reaction-mixture composition was that specified for the assay of phosphatase activity with pyridoxamine phosphate as described in the Experimental section. In Expt. 2, however, only 1·5 μmoles of each substrate was used. Approx. 0·1 mg. of protein was used in each case. To each 4·5 ml. of stopped reaction mixture was added 1·25 ml. of approx. N-H_2SO_4, and water to 8·0 ml. Inorganic phosphate was then measured by the method of Gomori (1941–42). The colorimetric blanks were the controls, in which NaOH was added to reaction mixtures before the substrate. A solution of KH_2PO_4 was used as standard.

	Inorganic phosphate formed (μmole)			Relative reaction velocity (initial)
Time interval (min.)	2·5	5	10	
Expt. 1				
Substrate				
Pyridoxamine phosphate	0·07	0·14	0·22	0·64
Pyridoxal phosphate	0·045	0·09	0·14	0·41
p-Nitrophenyl phosphate	0·11	0·19	0·31	1·00
Expt. 2				
Pyridoxamine phosphate	0·10	—	—	0·67
Pyridoxal phosphate	0·06	—	—	0·40
p-Nitrophenyl phosphate	0·15	—	—	1·00
Pyridoxamine and pyridoxal phosphates	0·06	—	—	0·40
Pyridoxal and *p*-nitrophenyl phosphates	0·06	—	—	0·40
Pyridoxamine and *p*-nitrophenyl phosphates	0·12	—	—	0·80

Table 4. *Relative reaction rates for pyridoxal, pyridoxamine and* p-*nitrophenyl phosphates with different enzyme preparations*

Reaction-mixture composition and assay procedure used were the same as described for Table 3. Approx. 0·1 mg. of enzyme protein and 2·0 μmoles of substrate were used and the concentration of $MgSO_4$ was 2·0 mM in each case. Enzyme A was prepared by Armour Laboratories, and enzyme B by Worthington Biochemical Corp.

	Inorganic phosphate formed (μmole)				Relative reaction velocity (initial)	
	Enzyme A		Enzyme B			
Time interval (min.) ...	2·5	5	2·5	5	Enzyme A	Enzyme B
Substrate						
Pyridoxamine phosphate	0·10	0·23	0·11	0·25	0·67	0·73
Pyridoxal phosphate	0·06	0·14	0·05	0·12	0·40	0·33
p-Nitrophenyl phosphate	0·15	0·33	0·15	0·34	1·00	1·00

and *p*-nitrophenyl phosphates, experiments were carried out with all three substrates, both singly and in pairs. The results are given as Expt. 2 in Table 3. Relative reaction velocities for the three substrates were also determined with an alternative preparation of alkaline phosphatase (Worthington Biochemical Corp.). Preliminary experiments showed that this sample of the enzyme had an absolute requirement for Mg^{2+} ions, and also suggested a lower pyridoxal phosphate-phosphatase activity when compared with activity with *p*-nitrophenyl phosphate. The results of an experiment to determine relative reaction velocities with each enzyme preparation in the presence of Mg^{2+} ions are given in Table 4.

DISCUSSION

In schemes summarizing vitamin B_6 metabolism (Snell, 1958; Braunstein, 1960), the hydrolysis of both pyridoxal and pyridoxamine phosphates by phosphatase action has been postulated. No evidence, however, has been published about the nature and specificity of the phosphatases involved. Whereas most enzyme reactions involved in vitamin B_6 metabolism appear to be specific for the heterocyclic-ring portion of the vitamin, the oxidation of pyridoxal to 4-pyridoxic acid has been shown to be catalysed by the broadly specific mammalian enzyme aldehyde oxidase (Schwartz & Kjeldgaard, 1951). From the results presented here, it appears that alkaline phosphatases of apparently broad specificity are involved in vitamin B_6 metabolism. Results obtained with pairs of mixed substrates (Table 3), where the observed activity was in all cases less than the sum of the individual activities, suggests that a mixture of narrowly specific phosphatases is not involved. The competitive inhibition by pyridoxamine phosphate of pyridoxal phosphate breakdown also supports this view. The slightly different relative reaction velocities observed with different sources of phosphatase preparation may mean that each preparation is a mixture of enzymes, each with comparatively broad but quantitatively different specificity.

K_m 0·05 mM for pyridoxal phosphate, compared with K_m 0·06 mM for *p*-nitrophenyl phosphate measured under identical conditions, appears to be of the same order as K_m values for other naturally occurring substrates as reported in the literature, although such comparison is difficult because of the influence of pH, buffers etc. (Roche, 1950). Activation by Mg^{2+} ions and inhibition by metal-chelating agents and orthophosphate are common phenomena with mammalian alkaline phosphatases (Roche, 1950). The apparent activation of pyridoxal phosphate hydrolysis by NH_4^+ ions, observed by Pogell (1958) with *Escherichia coli* extracts, could not be demonstrated in the present case; nor could the inhibition of phosphatase activity by NH_4^+ ions as reported by Aebi (1949) and Motzok & Wynne (1950).

The possible role of a phosphatase for the coenzyme pyridoxal phosphate in the control of amino acid metabolism has previously been pointed out (Turner & Happold, 1961).

SUMMARY

1. An intestinal-phosphatase preparation of bovine origin was found to hydrolyse pyridoxal phosphate. At a substrate concentration of 0·1 mM, the pH optimum is approx. 8·9, and at this pH value K_m is approx. 0·05 mM. K_m for *p*-nitrophenyl phosphate, a commonly used substrate for alkaline-phosphatase assays, was found to be 0·06 mM when measured under identical conditions.

2. Phosphatase activity was increased by Mg^{2+} ions, the optimum concentration being approx. 2 mM. The presence of Mg^{2+} ions did not affect the pH optimum. Versenol (*N*-hydroxyethylethylenediaminetriacetic acid) inhibited activity at all concentrations used. Both pyridoxamine phosphate and inorganic orthophosphate inhibited pyridoxal phosphate breakdown competitively, the K_i values being 0·15 and 0·06 mM respectively. Neither pyridoxal nor NH_4^+ ions affected the phosphatase activity.

3. The enzyme preparation also hydrolysed pyridoxamine phosphate at optimum pH 8·9. With the rate of hydrolysis of *p*-nitrophenyl phosphate used as a standard, the relative reaction velocities for pyridoxal and pyridoxamine phosphate were approx. 0·41 and 0·64 respectively. With an alternative preparation of alkaline phosphatase, similar relative reaction rates were observed.

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Studies on the Interaction of Zinc, Cadmium and Mercuric Ions with Native and Chemically Modified Human Serum Albumin

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The ions of zinc, cadmium and mercury vary widely in their effects on living organisms. Zinc ions are well tolerated in mammals, and distribution studies (Gilbert & Taylor, 1956) have shown that they are located in organs rich in enzymes, such as liver, kidney and testes. Zinc has been found essential to the function of alkaline phosphatase (Mathies, 1958), carboxypeptidase B (Folk, Piez, Carroll & Gladner, 1960), liver alcohol dehydrogenase (Vallee & Hoch, 1957), glutamic dehydrogenase (Adelstein & Vallee, 1958), muscle dehydrogenase (Vallee & Wacker, 1956) and leucocyte carbonic anhydrase (Vallee, Hoch & Hughes, 1954). It is also essential in lower organisms to enzymes such as yeast alcohol dehydrogenase (Vallee & Hoch, 1955) and hexokinase in *Neurospora crassa* (Medina & Nicholas, 1957). The requirement of zinc for these enzymes does not necessarily always imply direct zinc–protein linkage. Nevertheless, if such an interaction is involved, a detailed study of the interaction of Zn^{2+} ions with albumin over a range of pH should provide a model with which to compare the effect of pH on these enzymes.

Cadmium ions, although chemically similar to Zn^{2+} ions, are toxic to many organisms. They have been found, however, in the cortex of the normal horse kidney in a protein also containing Zn^{2+} ions (Kägi & Vallee, 1960).

Mercuric ions are almost universally toxic to living matter. This toxicity is associated with the high affinity for protein thiol groups.

The affinity of proteins for these three metal ions has led to their use as precipitants in plasma-fractionation procedures. Zinc ions were used by Ketz & Mahl (1955), Cd^{2+} ions by Tolok (1959) and Hg^{2+} ions by Astrup, Schilling, Birch-Anderson & Olsen (1954).

Though previous workers have studied the interactions of these ions with various soluble proteins and have postulated their sites of binding, there are no studies over a range of pH. In this paper quantitative studies are reported on the interactions of native human albumin and some of its chemical modifications with the ions of zinc, cadmium and mercury over the accessible pH range. The techniques used were equilibrium dialysis and radioactive-isotope dilution.

METHODS

Zinc. $^{65}ZnCl_2$ of approximate specific activity 100 mc/g. of Zn was obtained from The Radiochemical Centre, Amersham, Bucks. Carrier inactive $ZnSO_4$ was added to bring the specific activity to the required value.

Cadmium and mercury. Both these materials were obtained by neutron irradiation of inactive material in BEPO at A.E.R.E., Harwell. Cadmium metal (0·5 g.) was irradiated for 4 weeks at a neutron flux of 10^{12}/cm.^{2}/sec. Mercuric oxide (2 g.) was irradiated for 4 weeks at a neutron flux of $1{\cdot}2 \times 10^{12}$/cm.^{2}/sec. No significant quantities of isotopes of other elements are produced by these irradiations. Both samples were dissolved in warm 2N-HNO_3. Portions were diluted to the required concentration and activity before use.

Measurement of radioactivity. The γ-radiation of each isotope was used for isotope-dilution analysis. Samples (10 ml.) of each solution to be analysed were placed in an annular NaI crystal attached to a photomultiplier and an Ekco automatic scaler. Each sample was counted for a minimum of 160 000 counts.

Equilibrium dialysis. All dialyses were carried out with 10–14 ml. of 0·5–1·0% (w/v) protein solution inside Visking cellulose membranes. The outer solution (120 ml.) was of known pH and I 0·15. For most experiments the concentrations of the metal ions were (mg./l.): Zn^{2+}, 10; Cd^{2+}, 10; Hg^{2+}, 20. Each dialysis was done in a dialysis rocker overnight. Control experiments showed that with this apparatus 0·15M-NaCl soln. came into equilibrium with water in 3 hr.

Outer solutions. For the range pH 4–6 sodium acetate–acetic acid buffers were used. The range pH 6·5–8·5 was covered with either 0·15M-sodium acetate or mixtures of 0·15M-$NaHCO_3$ and 0·15M-NaCl.

Protein concentration. Protein was estimated by semi-micro-Kjeldahl analysis by using a protein:N factor of 6·25.

Albumins. Native human albumin was prepared from plasma by the cold-ether-fractionation technique of Kekwick & Mackay (1954). Chemically modified albumins were prepared by standard procedures as follows: acetylation, Marrack & Orlans (1954); deamination, Fraenkel-Conrat, Bean & Lineweaver (1949); esterification, Chibnall, Mangan & Rees (1958); bromoacetylation, Korman & Clarke (1956); bromopropionation, Korman & Clarke (1956), β-bromopropionic acid being substituted for bromoacetic acid mole for mole.

Analysis of modified albumins. Amide groups were determined by the method of Chibnall *et al.* (1958) and free amino groups by the method of Van Slyke (1929) as modified by Baddiley, Kekwick & Thain (1952).

Histidine was separated from the protein hydrolysates by the method of Moore & Stein (1951). The separated histidine peak was assayed by the method of Sluyterman (1960). Methoxyl groups were measured by the Zeisel method (Grant, 1951). Pepsin was obtained from The Armour Laboratories as a crystalline material from porcine mucosa.

RESULTS

The effect of pH on the binding of Zn^{2+} and Cd^{2+} ions to albumin is shown in Fig. 1. The pH of maximum binding was pH 7–8 for both ions. No significant quantitative difference was found between bovine and human albumins. Maximum binding for zinc was 8 ± 1 g.atoms/10^5 g. of protein, whereas for cadmium it was $4 \pm 0·5$ g.atoms/10^5 g. of protein. Neither ion was bound below pH 4. Mercuric ions reacted differently with albumins, both quantitatively and with respect to pH. Only in the range pH 2·5–4 were the complex systems soluble. At more alkaline pH values the Hg^{2+} ion was precipitated as its hydroxide. In the pH range used the binding was constant at 104 ± 2 g.atoms of mercury/10^5 g. of human albumin and 130 ± 2 g.-atoms/10^5 g. of bovine albumin.

Acetylation of human albumin increased the maximum ion-binding to $9·5 \pm 1·0$ g.atoms of zinc (Fig. 2) and $6·7 \pm 0·8$ g.atoms of cadmium/10^5 g. of protein. It was not possible to study Hg^{2+} ion-binding as this modified protein was insoluble in the pH range suitable for this ion. Deaminated albumin (Fig. 2) had similar but somewhat decreased ion-binding properties to acetylated albumin. Attempts

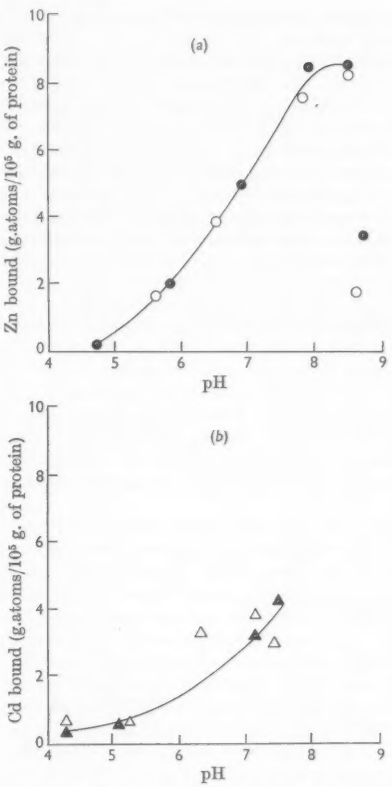

Fig. 1. Effect of pH on Zn^{2+} (*a*) and Cd^{2+} (*b*) ion-binding to human albumin (●, ▲) and bovine albumin (○, △).

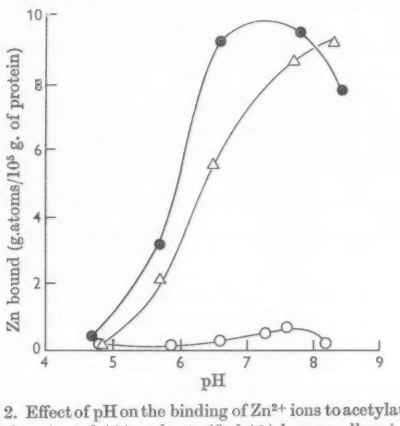

Fig. 2. Effect of pH on the binding of Zn^{2+} ions to acetylated (●), deaminated (△) and esterified (○) human albumins.

to eliminate by esterification the carboxyl groups as binding sites on the native albumin produced a protein with very low affinities for Zn^{2+} (Fig. 2) and Cd^{2+} ions. The maximum values found were $0{\cdot}7 \pm 0{\cdot}2$ g.atom of zinc and $0{\cdot}8 \pm 0{\cdot}2$ g.atom of cadmium bound/10^5 g. of protein at pH 7·5.

A derivative with unusual metal-ion-binding properties was prepared by reaction of bromoacetic acid with native albumin. This derivative had 36 ± 3 g.amino groups blocked by the reagent. It bound a maximum of 81 ± 2 g.atoms of zinc and 78 ± 2 g.atoms of cadmium/10^5 g. of protein at pH 6 (Fig. 3). At more alkaline pH the system was unstable and the Zn^{2+} ions were precipitated as the hydroxide. This protein derivative and its mercury complexes were sufficiently soluble to be studied over the range pH 2·9–5·8 (Fig. 3). Maximum Hg^{2+} ion-binding was 190 ± 4 g.atoms/10^5 g. of protein. A similar protein derivative with 63 ± 3 substituted amino groups was made with β-bromopropionic acid. This protein still had greater metal-ion-binding properties than the native material but less than the bromoacetic acid derivative (Fig. 3). The maximum binding values observed were 18 g.-atoms of zinc at pH 6·2 and 15 g.atoms of cadmium at pH 7·4/10^5 g. of protein. At more alkaline pH the metal ions began to be precipitated as their hydroxides. In the accessible range for Hg^{2+} ion-binding (pH 3·4–5·1) a similar effect was observed. The binding value rose from 1 to 7 g.atoms of mercury/10^5 g. of protein. The chemical analyses for the modified albumins used are shown in Table 1. A comparison of the variations of Zn^{2+} ion-binding of human albumin and pepsin is shown in Fig. 4.

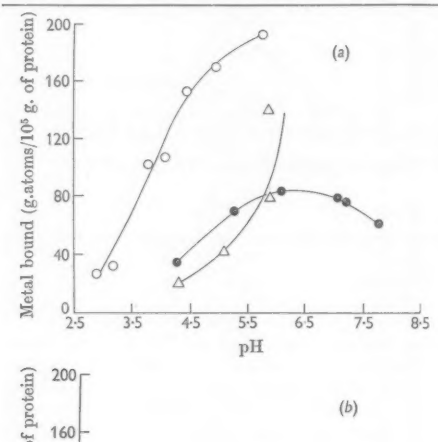

Fig. 3. Effect of pH on the binding of Zn^{2+} (●), Cd^{2+} (△) and Hg^{2+} (○) ions to (*a*) bromoacetic acid-treated albumin, and (*b*) β-bromopropionic acid-treated albumin.

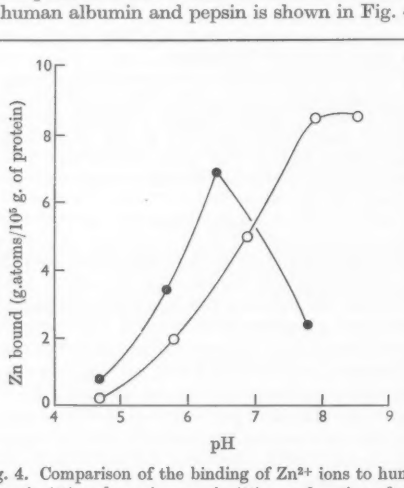

Fig. 4. Comparison of the binding of Zn^{2+} ions to human albumin (○) and porcine pepsin (●) as a function of pH.

Table 1. *Chemical analyses of native and chemically modified proteins*

For methods of preparation and analysis see text.

Preparation	Moles/10^5 g. of protein			
	Amide	Amino	Histidine	Methoxyl
Native human albumin	64±2	87±3	23±1	*
Acetylated albumin	66±2	32±3	23±1	*
Deaminated albumin	62±2	33±3	14±1	*
Esterified albumin	56±2	97±3	19±1	120
Carboxymethylated albumin	*	51±3	3±2	*
β-Carboxyethylated albumin	*	24±3	*	*

* Not determined.

DISCUSSION

The small numbers of Zn^{2+} and Cd^{2+} ions bound per 10^5 g. of albumin suggest that the number of sites available for binding is small. Many authors have concluded that the binding sites were the imidazole residues of histidine (Gurd & Goodman, 1952; Saroff & Mark, 1953; Gurd & Murray, 1954; Rao & Lal, 1958; Vodrazka, 1959; Waldmann-Meyer, 1960). Their evidence was obtained from equilibrium dialysis, polarographic, electrophoresis and chemical modification studies. These authors did not agree about the properties and numbers of sites per molecule. Saroff & Mark (1953) found eight uniform sites whereas Rao & Lal (1958) found two sites, which were different from the remainder in that they bound zinc preferentially to cadmium. Kacena (1954) found that there were 50 or more binding sites for zinc. Waldmann-Meyer (1960) found 17 sites per molecule of pK 7·1. He suggested that the sites of binding were the imidazole residues, because they had the correct pK and were equal in number to the histidine content of albumin. At first sight the results shown in this paper appeared to confirm the imidazole-binding-site hypothesis. The results obtained with acetylated, deaminated and esterified albumin (Table 1) showed, however, that this theory of binding was unsatisfactory. Deliberate chemical modification of the free amino groups of the protein enhanced the metal-binding of both Zn^{2+} and Cd^{2+} ions. The significant effect of acetylation on the protein was to block positively charged groups, so making the protein more electronegative. Deamination had a similar effect but also destroyed 40 % of the histidine residues in the native protein. Zinc and Cd^{2+} ion-binding was similar for these two preparations despite the difference in histidine content. Esterification of the carboxyl groups decreased metal-ion-binding to less than 1 g.ion/10^5 g. of albumin. In this preparation the histidine content was not significantly altered by the chemical modification of the protein. If the imidazole residues of the protein alone were responsible for Zn^{2+} and Cd^{2+} ion-binding, then this modification should have shown similar binding to native albumin. The values observed suggest that the single thiol group was responsible in these experiments. Kägi & Vallee (1960) showed that in the cadmium-containing metallothionein the thiol groups were the sites of binding.

To test the validity of the histidine-binding theory equilibrium-dialysis studies with Zn^{2+} ions and pepsin were tried. This protein had six histidine residues/10^5 g. of protein (Brand, 1948), compared with 23 for human albumin. The results (Fig. 4) did not show the expected difference between the two proteins. They did show the same relative change as the difference in free carboxyl groups, which number 140 for albumin and 105 for pepsin in each 10^5 g. of protein.

The results with pepsin and native and esterified albumins do not show a specific 1:1 carboxyl to metal-ion-binding. This implies an overall charge effect on the molecule rather than specific sites. Scatchard (1949) expressed the opinion that it was not necessary for the metal ion to react at the site from which the H^+ ion was displaced. He explained this by assuming that it was not the free metal ion which reacted with the protein, but a complex of metal ion and buffer. The combination of the ion with the buffer produced an electrostatic effect similar to the displacement of a H^+ ion. He concluded that buffered systems 'should not be used in the quantitative study of equilibria in protein solution'. The experimental difficulties when excluding buffers other than the protein itself preclude any extensive studies with metal ions. Moreover, in biological systems some additional buffer system is usually present. A further study of metal–protein complexes and the role of different buffer systems is required.

The pH for maximum binding was in the physiological range 7–8 for both Zn^{2+} and Cd^{2+} ions. This suggests that enzymes which need to combine directly with the metal ions in this pH range will be able to make maximum use of the ions available in the system. The present experiments did not show any unusual chemical properties to account for the toxicity of cadmium compared with zinc. But Kägi & Vallee (1960) showed that Cd^{2+} ions were bound in preference to Zn^{2+} ions to the thiol groups of metallothionein. This inversion of the preference for Zn^{2+} ions, shown by those polar groups of proteins other than thiols, suggests that cadmium toxicity is related to the thiol interaction. This reaction relates cadmium to mercury, which is known to have toxic effects due to its combination with thiol groups of proteins.

The species difference of bovine and human albumin with Hg^{2+} ions may be due either to spatial differences of the carboxyl groups or to hydrolysis of some amide groups of bovine albumin in the presence of Hg^{2+} ions. At present there is no evidence to explain these observations. Studies with the complexes of amino acids and Hg^{2+} ions (Perkins, 1952) showed that they had remarkable binding affinities. The high values for the number of ions bound reflected this property. The only numerically possible sites of interaction for Hg^{2+} ions were the carboxyl groups. The known high affinity of the thiol group for Hg^{2+} ions would have accounted for only 1 g.ion/10^5 g. of protein.

The present experiments showed that free Hg^{2+} ions would not exist at the pH of most biological systems. However, Saroff & Mark (1953) showed that the predominantly covalent mercuric chloride

combined with albumin at pH 7·5. It is well known that this reagent will react with thiol groups. Both the toxic metals cadmium and mercury thus had a greater affinity than zinc for the thiol groups of proteins. It is therefore probable that the functional groups responsible for the toxic action of the compounds of these two metals are the thiols, although any excess of metal ions will combine with other groups.

Lal & Rao (1957) have shown that Zn^{2+} ions can also combine with albumin as a complex. At pH 6·5 the complex bound was zinc acetate. It is probable that in biological systems in which proteins and metal ions interact, account must be taken of the buffer systems other than protein present. As yet there is no clear evidence to show whether the metal–enzyme systems are examples of the general metal-ion-binding shown by albumin or whether there are unusual prosthetic groups conferring more specific properties.

Treatment of human albumin with bromoacetic acid and β-bromopropionic acid greatly enhanced the ion-binding properties for all three metal ions studied (Fig. 3). The bromoacetic acid-treated protein was the more potent and was also capable of acting over a wider range of pH. Korman & Clarke (1956) showed that bromoacetic acid combined with the amino groups of proteins to produce derivatives of the structure $-N(CH_2 \cdot CO_2H)_2$. This structure is half of the well-known metal-chelating agent ethylenediaminetetra-acetic acid. Schwarzenbach & Freitag (1951) showed that the log stability constants for the zinc and cadmium complexes of this acid were 16·5 and 16·4 respectively. It seems probable that similar constants can be applied to the modified protein, as the zinc- and cadmium-binding values are little different. The only reactive groups present in proteins with similar constants are the thiols. Kägi & Vallee (1960) obtained values of 17 and 14 for the stability constants of the complexes with Cd^{2+} and Zn^{2+} ions respectively with metallothionein. Both the bromoacetic acid- and bromopropionic acid-modified albumins may be of value in introducing or transporting metal ions in systems where the free metal ions would be precipitated.

SUMMARY

1. Zinc and Cd^{2+} ions combine with native human and bovine albumins on similar or identical sites.

2. At pH 2·5–4·0, 130 g.ions of mercury combine with 10^5 g. of bovine albumin and 100 g.ions with 10^5 g. of human albumin.

3. Evidence is produced to suggest that the binding of Zn^{2+} and Cd^{2+} ions to native albumin is governed by the electronegative charge on the molecule.

4. The metal-ion-binding properties of albumins treated with bromoacetic acid and bromopropionic acid are reported.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **405th Meeting** *of the Society was held in The University of Birmingham, Edgbaston, Birmingham, 15, on Friday and Saturday, 28 and 29 April* 1961, *when the following papers were presented:*

COLLOQUIUM ON 'METABOLIC PATHWAYS OF FOREIGN COMPOUNDS'

The Metabolism of 2-(β-Methoxyethyl)pyridine

By W. A. M. Duncan and B. Scales. (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Research Department, Alderley Park, Macclesfield, Cheshire*)

After subcutaneous administration 2-(β-methoxyethyl)pyridine is rapidly distributed throughout the tissues and body fluids; 24 hr. later only very low concentrations of the compound are found in the animals. In the five animal species examined a maximum of 1·5% of the administered dose can be accounted for in the urine as unchanged drug.

During the 48 hr. following injection of a calf with 400 mg. of the drug/kg. no significant increase was observed in the urinary excretion of glucuronic acid, ethereal sulphate or reducing substances, nor was any evidence obtained for the presence of any metabolites containing a hydroxylated pyridine nucleus. A large increase in the amount of material which will chelate with copper was found. This metabolite has been isolated and identified as pyridine 2-acetic acid; in the calf and the four other species this compound accounts for 75–85% of the administered dose.

Using the Wilzbach technique 2-methylpyridine was tritiated, and from the purified product 2-(β-methoxyethyl)pyridine, generally labelled in the pyridine nucleus, was prepared. This material is now being used to determine the fate of the remaining 15–25% of the administered drug. An investigation of the metabolic pathway and enzyme systems involved is also being made.

The Metabolism and Toxicity of Arylthioureas

By R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

The conversion, $-NH\cdot CS\cdot NH \rightarrow -NH\cdot CO\cdot NH-$ *in vivo* is a reaction of considerable interest but the mechanism is unknown. This reaction probably occurs in liver microsomes, for Spector & Shideman (1959) have shown that thiopentone is converted into pentobarbitone in these particles, a process which is inhibited by SKF 525A. The thioureylene group also occurs in the arylthioureas, and we became interested in the metabolism of these compounds because of the remarkable difference in toxicity to rats between 1-phenyl-2-thiourea (LD_{50} 5 mg./kg.) and 1:3-diphenyl-2-thiourea (LD_{50} > 2000 mg./kg.). Phenylthiourea is also highly toxic to rabbits (LD_{50} about 40 mg./kg.), while diphenylthiourea is relatively non-toxic. It was soon discovered that phenylthiourea was readily desulphurized in rats and rabbits, whereas diphenylthiourea was not. Toxicity might therefore be related to the desulphuration process.

On feeding to rabbits, diphenylthiourea was found to be converted into 4-hydroxydiphenylthiourea and excreted mainly as a glucuronide which was isolated (Smith & Williams, 1961*a*). Diphenylurea and its derivatives were not found. The metabolism of phenylthiourea was studied with 2-^{14}C-, ring-^{14}C- and ^{35}S-labels and the metabolites were found to be, unchanged phenylthiourea (6%), *p*-hydroxyphenylthiourea (16%), phenylcyanamide (1%), phenylcarbamic acid (30%, conjugated with glucuronic acid), phenylurea (4%), 4-hydroxyphenylurea (14%), aniline (4%), urea (3%) and sulphate (62%) (Scheline, Smith & Williams, 1961). The results were checked by feeding all the intermediate compounds, none of which was highly toxic. The results suggest that the main initial reaction of phenylthiourea was

$$C_6H_5NH\cdot CS\cdot NH_2 \rightarrow C_6H_5NH\cdot CN + H_2S.$$

On feeding phenylcyanamide, it was found that the main metabolite was phenylcarbamic acid ester glucuronide and a small amount of phenylurea and 4-hydroxyphenylurea. On feeding sodium phenylcarbamate, the main metabolite was the corresponding glucuronide. Phenylurea was converted mainly into 4-hydroxyphenylurea. It appeared that less than 20% of phenylthiourea was converted into phenylurea and that the conversion occurred thus:

$$C_6H_5NH\cdot CS\cdot NH_2 \xrightarrow{-H_2S} C_6H_5NH\cdot CN \xrightarrow{+H_2O} C_6H_5NH\cdot CO\cdot NH_2.$$

The main metabolic route of phenylcyanamide was probably

$$C_6H_5NH\cdot CN \rightarrow C_6H_5\cdot NH\cdot COOH \rightarrow C_6H_5\cdot NH\cdot COOC_6H_9O_6.$$

Since none of the aromatic metabolites of phenylthiourea was highly toxic, toxicity could be due to release of H_2S in the tissues. Aqueous solutions of pure H_2S were found to be highly toxic to rats, the LD_{50} being about 0·27–0·55 mg./kg. by intravenous injection. Calculations showed that one LD_{50} of phenylthiourea could release 0·7 mg. of H_2S/kg.

Rats could be protected against phenylthiourea by injecting 1-methyl-1-phenylthiourea just before phenylthiourea. 1-Methyl-3-phenylthiourea and 2-methyl-1-phenyl*iso*thiourea afforded no protection. By feeding [^{35}S]phenylthiourea with and without 1-methyl-1-phenylthiourea, it could be shown that the methyl derivative cause a decrease in the amount of ^{35}S excreted as sulphate. Thus the methyl derivative appeared to inhibit the desulphuration of phenylthiourea. The methyl derivative was not highly toxic, but it was almost completely desulphurized *in vivo*, possibly at a much slower rate than phenylthiourea (Smith & Williams, 1961*b*).

Other mono-aryl and diaryl-thioureas were studied and the results appeared to support the above conclusions.

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Aspects of the Formation of 'Foreign' Glucuronides in Developing Tissues

By G. J. Dutton. [*Department of Biochemistry, Queen's College, Dundee* (*University of St Andrews*), *Scotland*]

One major metabolic pathway of 'foreign' compounds is their conjugation, in the liver of various species, with glucuronic acid. This can occur by glucuronyl transfer from uridine diphosphate glucuronic acid (UDPglucuronic acid), being catalysed by UDPtransglucuronylase, an enzyme appearing in the 'glycogen-heavy microsome' fraction of homogenates. Such a pathway exists also in kidney, skin and gastrointestinal tract. 'Endogenous' glucuronides are formed similarly, and though the enzyme has recently been reported solubilized, there is no direct evidence yet of separate UDPtransglucuronylases for various classes of aglycones; the enzyme activity observed can be increased, under differing conditions, by several procedures. Certain glucuronides are reported to be formed also by transglucuronylation from existing glucuronides, involving β-glucuronidase or similar enzyme.

It is becoming clear that embryos may differ markedly in their biochemical pattern from adults or young animals. This contribution deals principally with observed pathways of 'foreign' glucuronide formation in some embryonic and infant tissues. Interpretation of results from such tissues requires care for, apart from the factors influencing observation of UDPtransglucuronylase activity, there is variation in composition and in relative size of developing organs. Degree of dependence upon maternal enzymes obviously must also be taken into account.

In the avian egg, the isolated embryo might offer an interesting comparison with the mammalian foetus, and recent work described from this laboratory includes the following of UDPtransglucuronylase activity in the formation of *o*-aminophenyl glucuronide in chick liver, from 9-day incubated embryo to adult bird, using UDPglucuronic acid-fortified homogenates. Preliminary experiments indicated that activity was present at 9 days and increased to about the 16th day; it then fell, to the 21st day. After hatching, levels rose, very rapidly at first, to higher values. The fall in activity was not due to inhibition and corresponded approximately with the onset of the 'cholesterol-fatty liver' observable in chick embryos. Low visible *o*-aminophenol conjugation over this period was also seen with chick-embryo-liver slices; otherwise, conjugation in these slices, unlike that appearing in corresponding mammalian preparations, did not clearly reflect results on UDPtransglucuronylase and UDPglucose dehydrogenase activity levels. Possible reasons for these observations were discussed.

Dihydrodiols as Metabolic Intermediates in Higher Animals

By R. P. Hopkins and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Certain cyclic hydrocarbons are known to give rise to dihydrodiols *in vivo*, e.g. naphthalene has been shown to be converted into 1:2-dihydronaphthalene-1:2-diol in several animal species (Young, 1947; Booth & Boyland, 1949; Corner & Young, 1954). It has been suggested that dihydrodiols may undergo dehydration, dehydrogenation and ring-fission, and may thereby serve as intermediates in the metabolic formation of monohydric phenols, dihydric phenols, quinones and dicarboxylic acids (Boyland, 1950; Smith, 1950; Young, 1950).

Examination of the urine of rats dosed with 1:2-dihydronaphthalene-1:2-diol (Boyland & Wiltshire, 1953; Corner & Young, 1955) has provided evidence that in the intact animal this dihydrodiol undergoes dehydration and dehydrogenation. Recently, Ayengar, Hayaishi, Nakajima & Tomida (1959) have demonstrated the occurrence in rabbit liver of a dehydrogenase system which converts *cyclo*hexa-1:3-diene-5:6-diol into catechol, and which also oxidizes 1:2-dihydronaphthalene-1:2-diol, presumably with the formation of 1:2-dihydroxynaphthalene, a compound which has been shown by Corner & Young (1954) to be a metabolite of naphthalene in the guinea pig.

Although dihydrodiols may serve as intermediates in ring-fission reactions in micro-organisms (Fernley & Evans, 1958), this does not appear to have been demonstrated in higher animals, and recently Tomida & Nakajima (1960) have reported that *cyclo*hexa-1:3-diene-5:6-diol, when administered to rabbits, is not converted into muconic acid. In a study of the metabolism of acenaphthylene (Brooks, Hopkins & Young, 1959) it was shown that in the rabbit this hydrocarbon gives rise to *cis*-acenaphthene-1:2-diol and to a mixture of (+)- and (−)-*trans*-acenaphthene-1:2-diol with a preponderance of the (+)-form. 1:8-Naphthalic anhydride was separated from the urine of rabbits and rats dosed with acenaphthylene (Hopkins, Brooks & Young, unpublished results) and in view of the ease with which naphthalic acid passes into the anhydride form it is reasonable to suppose that naphthalic acid is a metabolite of acenaphthylene. The possibility that 1:8-naphthalic acid arises from oxidation of the dihydrodiols formed in the metabolism of acenaphthylene has now been investigated by the administration of *cis*-acenaphthene-1:2-diol and (±)-*trans*-acenaphthene-1:2-diol to rats by stomach tube and by subcutaneous injection. In every case 1:8-naphthalic anhydride was separated from the urine. The significance of these observations with respect to the possible role of dihydrodiols in ring-fission reactions in the animal body was discussed.

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Mercapturic Acid Conjugation

By E. Boyland. (*Chester Beatty Research Institute: Institute of Cancer Research, Royal Cancer Hospital, London, S.W.* 3)

Many foreign organic compounds, some aliphatic (i.e. propyl bromide) and some aromatic (i.e. naphthalene), are excreted as mercapturic acids which are derivatives of acetylcysteine. The metabolites of purely aromatic compounds such as naphthalene or bromobenzene are usually *S*-(αβ-dihydro-β-hydroxy-α-phenyl)acetylcysteine compounds. Such derivatives decompose in cold acid solution when water is eliminated to give *S*-phenylacetylcysteine derivatives. The metabolism of these aromatic compounds involves oxidation to hypothetical intermediates with the properties of epoxides. These epoxides may react with water to give αβ-dihydro-αβ-dihydroxy derivatives or 'diols', or with cysteine derivatives to give metabolites of the mercapturic acid type. Compounds which contain chemically reactive groups such as the epoxide group of 1:2-epoxy-1:2:3:4-tetrahydronaphthalene or the chloro group of benzyl chloride react directly with the cysteine derivatives.

The formation of mercapturic acid in the rat involves the reaction of a chemically active or biochemically-activated foreign compound with glutathione in the liver. [The glutathione derivatives and other intermediates are excreted in bile but not in urine (Boyland, Ramsay & Sims, 1961)]. The glutathione derivatives appear to be converted into the corresponding cysteine derivatives in the kidney and the final reaction which occurs in the liver is the acetylation of the cysteine residue to yield the mercapturic acid which is excreted in urine. An enzyme, 'glutathione kinase', present in the supernatant fraction of rat-liver homogenate catalyses the reaction of organic compounds with glutathione, but not with cysteine, acetylcysteine or any other sulphydryl compounds (Booth, Boyland & Sims, 1961).

The aromatic amines aniline and 2-naphthylamine are excreted as mercapturic acids by rats. 2-Naphthylamine is apparently converted into 2-amino-1-naphthyl mercapturic acid and also into *S*-(2-amino-5:6-dihydro-6-hydroxy-5-naphthyl)-*N*-acetylcysteine among many other products (D. Manson, personal communication). The first compound may arise by oxidation of 2-naphthylamine to 2-naphthylhydroxylamine (RNHOH) which can react with glutathione (GSH) as a quinolimide to yield *S*-(2-amino-1-naphthyl)glutathione which is converted into 2-amino-1-naphthyl mercapturic acid which is excreted. The biosynthesis of the other mercapturic acid derivative from 2-naphthylamine probably arises through the formation of the

hypothetical 2-acetamino-5:6-epoxy-5:6-dihydronaphthalene which then reacts with glutathione.

Mercapturic acid conjugation is a general reaction by which foreign compounds which are either chemically reactive (i.e. benzyl chloride) or insoluble (i.e. naphthalene and phenanthrene) are converted into inert water-soluble ionized compounds which can be excreted by the kidney. There are probably some types of foreign compounds which are conjugated as mercapturic acids still to be discovered.

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The Metabolism of some Bromo Paraffins

By Sybil P. James. (*Department of Physiology, The Medical School, University of Birmingham*)

The biological formation of *N*-acetyl-*S*-alkylcysteines from members of the bromo paraffin series was reported simultaneously by Thomson, Maw & Young (1958) for 1-bromoethane and by Bray & James (1958) for 1-bromo-butane, -pentane, -hexane, -heptane and -octane. The formation of mercapturic acid in the animal body from bromobutane was first suspected from the intense nitroprusside colour given after urines of rabbits or rats dosed with bromobutane had been hydrolysed with alkali; the presence of butylmercapturic acid was demonstrated on paper chromatograms and comfirmed by isolation (Bray & James, 1960). Urines obtained from rabbits and rats dosed with 1-bromo-pentane, -hexane, -heptane and -octane similarly gave after hydrolysis with NaOH a colour with sodium nitroprusside which diminished in intensity as the series was ascended. Although measurement of this colour suggested that relatively large amounts of mercapturic acid were formed, the isolation of the acids proved difficult and only the pentyl- and hexyl-mercapturic acids were isolated. Paper chromatography showed that more than one sulphur-containing metabolite was present in the urines. Ether extraction separated the metabolites of bromobutane since only the mercapturic acid was readily soluble in ether. This gave an indication of the maximum percentage of the dose which could be excreted in this form. In the higher members of the series this method could not be used as all the sulphur-containing metabolites were soluble in ether. The amounts of mercapturic acid excreted have now been estimated by measuring the areas of the mercapturic acid spots produced on spraying paper chromatograms of urine samples with potassium dichromate and silver nitrate (Knight & Young, 1958). This specific method shows that only a small proportion of the 'apparent mercapturic acid' as measured by the nitroprusside colour is, in fact, mercapturic acid. Not more than a trace of heptylmercapturic acid is present in the urines of rabbit or rat dosed with bromoheptane and no octyl mercapturic acid was detected in the urines of animals dosed with bromo-octane.

The nature of the other sulphur-containing metabolites of the bromo paraffins is still under investigation. No sulphoxide of butylmercapturic acid has been detected [Thomson & Young (1960) found a sulphoxide of ethylmercapturic acid.] Purification of the ether-insoluble metabolite from bromobutane gives a product which contains bivalent sulphur and which does not react with ninhydrin. On hydrolysis with N-HCl a ninhydrin-reacting sulphur-containing substance is formed which yields butylcysteine on hydrolysis with 6N-HCl. The nature of this metabolite was discussed.

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COMMUNICATIONS

(1) The Metabolism of [1-^{14}C]Glycerol by Rat-Intestine Slices *In Vitro*

By D. R. Saunders and A. M. Dawson (introduced by B. H. Billing) (*Medical Unit, Royal Free Hospital, London, W.C.* 1)

Free glycerol, whether fed or liberated from dietary fat in the small intestine, is poorly used by the intestinal mucosa for glyceride synthesis (Gidez & Karnovsky, 1954). These observations are supported by experiments *in vitro* with homogenate systems of small intestine mucosa (Buell & Reiser, 1959; Clark & Hübscher, 1961) in which there is an apparent lack of glycerokinase. Thus, glycerol is assumed to be metabolically inert in the intestinal mucosa, for initial phosphorylation is essential in current schemes of mammalian glycerol metabolism (Kennedy, 1957). As the experiments *in vivo* by Buchs & Favarger (1959) cast some doubt on these assumptions, we have studied the problem *in vitro*

by incubating tracer amounts of ^{14}C substrates with everted segments of rat small intestine.

After incubation for 1 hr. at 25°, approximately 12% of tracer [1-^{14}C]glycerol is metabolized to CO_2 and lipid. This metabolic activity resides in the mucosa rather than muscularis and cannot be accounted for by contaminating bacteria. Furthermore, under conditions in which it is known that fatty acids are transported into the mucosal cell and esterified (Dawson & Isselbacher, 1960), there is an increased incorporation of label into lipid; this increase is of the same magnitude as when [U-^{14}C]glucose is the tracer substrate.

When [1-^{14}C]glycerol is the substrate, about 90% of the lipid radioactivity is in glyceride-glyceride. When the latter is oxidized, and the C-1 and C-3 atoms isolated as the formaldimedone (Reeves, 1941), all the radioactivity is recovered. This suggests that the carbon chain of glycerol is incorporated intact into glyceride.

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(2) Enzymically Active Sub-units of L-Myosin

By H. Mueller and S. V. Perry. (*Department of Biochemistry, University of Birmingham*)

Previously it has been shown (Mueller & Perry, 1960, 1961) by chromatography on diethylaminoethylcellulose that two enzymically active fractions can be isolated from the standard preparations of heavy meromyosin prepared by tryptic digestion (Szent-Györgyi, 1953). Extension of these studies to the corresponding subunit of similar solubility properties obtained by digestion with chymotrypsin (Gergely, Gouvea & Karibian, 1955) has revealed that a similar fractionation of enzymic activity can also be made in this case. Whereas with tryptic digestion the enzymically active fraction eluted at an ionic strength lower than 0·17 consistently represented 10–15% of the whole, the comparable fraction from the chymotrypsin-prepared heavy meromyosin often represented a more variable fraction of the original meromyosin. The more readily eluted fractions obtained in both these studies may represent fragments resulting from further degradation of the main component present in heavy meromyosin preparations. This view is supported by the finding that tryptic digestion of heavy meromyosin could be carried out with little loss in its adenosine triphosphatase activity and an increase in the fraction eluted at ionic strength lower than 0·17 at pH 7·6 from diethylaminoethylcellulose. The alteration in chromatographic properties observed during tryptic digestion of heavy meromyosin was correlated with changes in the ultracentrifugation pattern occurring during the process.

The apparent similarity in the chromatographic behaviour of the heavy meromyosins obtained by tryptic and chymotryptic digestion was correlated with the appearance of these subunits on electrophoresis on starch gel in 8M-urea under the conditions used by Smithies (1955). The appearance of both light and heavy meromyosins was different from that of L-myosin, which gave one main band accompanied by 3–4 minor bands under the same conditions. The general appearance of the light meromyosins obtained by the two enzymic methods was characterized by certain common features, although some differences could be recognized. Similar conclusions applied to the heavy meromyosins.

These investigations indicate that a variety of enzymically active fragments may be obtained by proteolytic digestion of the L-myosin molecule, although certain broad similarities can be observed in the fragments obtained on limited digestion by trypsin and chymotrypsin (cf. Middlebrook, 1959).

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(3) The Sulphatases of *Patella vulgata*

By P. F. Lloyd and K. O. Lloyd. (*Department of Chemistry, University College of North Wales, Bangor, Caerns*)

The liberation of inorganic sulphate was shown, by a qualitative turbidimetric ($BaSO_4$) method, to occur when certain carbohydrate sulphates were incubated with macerates of the visceral humps from limpets (Lloyd & Lloyd, 1958). Liberation of sulphate from D-glucose 6-sulphate (Dodgson & Spencer, 1954), D-galactose 6-sulphate, L-fucose sulphates, chondroitin sulphate and fucoidin was

effected by the enzyme preparation. These results have now been confirmed by quantitative studies using enzyme concentrates. Carrageenin and the mucilages from *Dilsea edulis* and *Porphyra umbilicalis* were not de-esterified, nor was keratosulphate when it was completely free from chondroitin sulphate, (Lloyd & Lloyd, 1960).

Considerable purification of the limpet sulphatase was achieved by salt and acetone fractionation. A 115-fold increase in activity was thus achieved, the test substrate used being the L-fucose monosulphate fraction prepared by sulphation of the sugar with pyridine–SO_3. This enzyme concentrate was also active towards D-glucose 6-sulphate, D-glucose 3-sulphate, D-galactose 6-sulphate, chondroitin sulphate and fucoidin.

Prolonged incubation of L-fucose monosulphates with the enzyme led to 75% desulphation. Disulphates of D-glucose and D-galactose were enzymically hydrolysed in part to the parent sugar, whereas treatment of a mixture of the di- and trisulphates of L-fucose with the enzyme yielded L-fucose, mono- and disulphates but no L-fucose. These data might suggest that the enzyme specifically attacks sulphate-ester groups located at one position only of the fucose molecule.

The micro-benzidine method (Dodgson & Spencer, 1953) was unsuitable for the assay of the limpet (L-fucose) sulphatase since it gave low values for liberated sulphate. A new assay method was therefore developed which consisted of passing the incubation mixture through a short column of De-Acidite FF (acetate form) to remove unhydrolysed monosaccharide sulphate and estimating the free sugar in the eluent by a colorimetric method (cysteine–sulphuric acid or phenol–sulphuric acid).

The limpet sulphatase has been characterized and its complete separation from aryl sulphatase achieved by chromatography on diethylaminoethylcellulose (eluting at pH 6 with 0·002M → 0·1M-citrate buffer or 0·01M → 0·2M-phosphate buffer). The separation was, however, attended by considerable deactivation of the enzymes.

The sulphatase mixture from limpet was more active against acid-degraded fucoidin and the mono- and oligo-saccharides derived from fucoidin by graded hydrolysis, than it was against the native polysaccharide.

We thank Professor Stanley Peat, F.R.S., for his interest and encouragement.

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(4) The Metabolism of 1:4-Quinones and their Reactivity with Sulphydryl Groups

By H. G. Bray and A. J. Garrett. (*Department of Physiology, The Medical School, University of Birmingham*)

Many compounds have been shown to react with sulphydryl groups and to form mercapturic acids *in vivo* (e.g. Barnes, James & Wood, 1959). 1:4-Quinones which are unsubstituted in at least one position of the quinone ring are known to react readily with sulphydryl groups but, except for menadione, studies of their metabolism are few (e.g. Glock, Thorp, Ungar & Wien, 1945).

An investigation has been made of some aspects of the metabolism in the rabbit of 2:5-dimethyl-1:4-benzoquinone (*p*-xyloquinone), 2-methyl-1:4-naphthoquinone (menadione), 3-hydroxy-2-methyl-1:4-naphthoquinone (phthiocol) and 2:3-dimethyl-1:4-naphthoquinone: the acute toxicity of other 1:4-quinones has precluded an extensive study. Only very small amounts of the unchanged quinones were excreted in the urine and the chief metabolites were glucuronic acid and sulphuric acid conjugates of the corresponding quinols. Chromatographic analysis has also revealed the presence of traces of phthiocol (cf. Shemiakin & Schukina, 1944) and a purple pigment (cf. Richert, 1951) as metabolites of menadione. A similar pigment has been detected as a metabolite of phthiocol. In no case was mercapturic acid formation detected.

Glutathione derivatives of several quinones have been prepared and the hydrolysis of *S*-(2-methyl-1:4-naphthoquinon-3-yl)glutathione by glutathionase preparations from guinea-pig liver has been demonstrated. Some possible reasons for the failure of quinones to form mercapturic acids were discussed.

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(5) The Action of Penicillins on Cell-wall Mucopeptide Synthesis in Sensitive and Resistant Strains of *Staphylococcus aureus*

By H. J. Rogers and J. Jeljaszewicz.* (*National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7*)

When *Staphylococcus aureus* is exposed to benzylpenicillin and some other antibiotics, the formation of cell-wall mucopeptide is inhibited

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(Park, 1958; Mandelstam & Rogers, 1959), and such inhibition has been proposed as the cause of the lethal effect of these substances (Park & Strominger, 1957). Although this hypothesis is plausible, little work has been done to make it secure by relating the concentrations of antibiotics required to kill the organisms to those required to inhibit mucopeptide synthesis.

In the present work the concentrations of benzylpenicillin and the new penicillins, phenoxymethylpenicillanic acid (celbenin, AB 1241, methicillin), 6-(α-phenoxypropionamido)penicillanic acid (Broxil), and 6-aminopenicillanic acid, required to inhibit mucopeptide synthesis have been compared with the lethal concentrations, using sensitive and resistant strains of *S. aureus*.

The system used for investigating mucopeptide synthesis was that described by Mandelstam & Rogers (1959) and Rogers & Perkins (1960). The *S. aureus* strain Oxford was used as the sensitive strain. Two resistant strains were used; one was derived by selection in the presence of benzylpenicillin from strain Oxford, and the other was strain 524/SC. The former did not produce penicillinase, whereas the latter did. Concentrations of about 0·2–0·3 μg./ml. (0·3–0·5 i.u./ml.) of benzylpenicillin inhibited the incorporation of either [1-^{14}C]glutamate, [1-^{14}C]alanine, [1-^{14}C]glycine or [U-^{14}C]lysine into the cell walls of the sensitive strain by about 70%. This inhibition was fully expressed about 10–20 min. after application of the antibiotic; no effect was detected for the first 10 min. About 50–100 times as much antibiotic was required to inhibit mucopeptide synthesis by the resistant substrain of *S. aureus* Oxford. The relative lethal effect of the new penicillins, celbenin, Broxil and 6-aminopenicillanic acid is known to differ by 1000-fold with sensitive strains of the organism. The order of effectiveness is benzylpenicillin = Broxil > celbenin ⩾ 6-aminopenicillanic acid. The same range of variation was found in concentrations required to inhibit mucopeptide synthesis; the effectiveness of the antibiotics as inhibitors was in the same order. The Oxford strain, which was resistant to benzylpenicillin, was not resistant to celbenin but was resistant to the other two new penicillins as far as mucopeptide biosynthesis was concerned. The penicillinase-producing strain 524/SC was as sensitive to celbenin as the sensitive Oxford strain, and the degree of inhibition reached was 80–90%. This result differs from the much lower result previously obtained (Mandelstam & Rogers, 1959) with high concentrations of benzylpenicillin.

Examination of the cell walls from the resistant substrain of *S. aureus* Oxford showed that they had the same composition as those prepared from the sensitive strain.

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(6) Further Studies of the Metabolism of Cinnamic Acids in Higher Plants

By J. B. Harborne and J. Janet Corner. (*John Innes Institute, Hertford, Herts*)

Previous work (Harborne & Corner, 1960) has established that cinnamic acids fed to plant tissues are usually converted into the glucose esters. Certain cinnamic acids in some plants undergo other conversions. Thus, examples of (1) *m*-hydroxylation (*p*-coumaric acid → 1-caffeoylglucose), (2) *o*-hydroxylation followed by ring closure and glycosylation (caffeic acid → aesculin) and (3) *O*-glycosylation (caffeic acid → caffeic acid 3- and 4-β-glucosides) have been detected. The purpose of this paper is to report three new metabolic paths, namely esterification with gentiobiose, *p*-hydroxylation and methylation.

Esterification with one, two and (in one case) three glucose residues occurs when *o*-coumaric or *m*-methoxycinnamic acids are fed to tomato leaves. The esters formed are probably 1-substituted gentiobioses. Under similar conditions, *p*-coumaric and caffeic acids are converted into their glucose esters but no detectable quantities of other sugar esters are formed. *p*-Hydroxylation occurs when cinnamic acid itself is fed to a variety of plants; the main product is 1-*p*-coumarylglucose, accompanied by some 1-ferulylglucose but only traces of 1-cinnamylglucose. L-Phenylalanine is also rapidly converted in some plants (e.g. tomato) but not in others (e.g. radish) into 1-*p*-coumarylglucose. Presumably an intermediate in this reaction is cinnamic acid, formed directly from phenylalanine by enzymatic deamination (Koukol & Conn, 1961).

p-Hydroxylation is also involved in the metabolism of *m*-coumaric acid. Some 1-*m*-coumarylglucose is formed, but the main products are caffeic acid 4-β-glucoside and 1-ferulylglucose. That methylation must occur after *p*-hydroxylation is indicated by the fact that *m*-methoxycinnamic acid does not serve as a precursor of ferulic acid. Methylation is also involved in the metabolism of caffeic acid in *Datura knightii* leaves; the major product is scopolin. This unexpected conversion has not been observed in any other of the plants examined, but a similar path is probably followed when scopolin is synthesized in potato tissue in response to viral and fungal infection (Andreae, 1948; Hughes & Swain, 1960) and in tobacco

as a result of callus formation (Sargent & Skoog, 1960).

Although many enzymes are clearly involved in these transformations, few have yet been isolated, or studied in detail. While most of the enzymes clearly function in polyphenol biosynthesis (see McCalla & Neish, 1959) others may only be present in the plant for detoxification purposes.

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(7) The Distribution of Albumin Synthesis among the Morphological Constituents of the Microsome Fraction from Rat Liver

By Alexandra von der Decken* and P. N. Campbell. (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W.* 1)

There have been many studies on the protein synthetic activity of the isolated 'microsome' fraction from rat liver (see Campbell, 1960). More recently evidence has been obtained for the synthesis of serum albumin by such a fraction (Campbell, Greengard & Kernot, 1960). However, even on visual examination it is apparent that such a microsome pellet is not homogeneous. An attempt has therefore been made to subfractionate the morphological constituents of the microsome fraction and to determine their characteristics for protein synthesis.

A homogenate obtained from the liver of starved rats 48 hr. after partial hepatectomy was centrifuged for 20 min. at 12 000*g*. The supernatant was then centrifuged for 15 min. at 125 000$g_{av.}$ in the SW39 rotor of the Model L Spinco preparative centrifuge. The residue provided a 'heavy' microsome fraction. The supernatant was centrifuged for 60 min. at 105 000$g_{av.}$ to provide a 'light' microsome fraction. An unfractionated microsome preparation was obtained by centrifuging a portion of the 12 000*g* supernatant at 105 000*g* for 60 min. and collecting the residue.

Electron microscopy indicated that the light microsome fraction consisted almost entirely of smooth endoplasmic reticulum and free electron-opaque particles, while the heavy fraction contained a wide variety of subcellular constituents.

Portions of each of the three microsome preparations were incubated for 30 min. at 37° in a medium containing 2-amino2-hydroxymethylpropane-1:3-diol buffer (Rendi & Hultin, 1960) with [^{14}C]leucine, adenosine triphosphate, and cell sap with and without phosphoenolpyruvate and pyruvate kinase. After incubation the particulate material was separated by centrifuging and the albumin released by ultrasonic vibrations. The radioactivity of this albumin and also that present in the soluble part of the incubation mixture was determined by the use of a specific antiserum. The radioactivity of the other soluble and insoluble proteins precipitated by trichloroacetic acid was also determined.

A comparison of the radioactivity of the insoluble microsomal proteins indicated no marked difference between the three preparations for their ability to incorporate amino acid into protein. The extent of microsomal albumin synthesis in the unfractionated and heavy preparations was similar but in the light fraction it was markedly less. In contrast the light fraction was the most active for the synthesis of soluble protein, including albumin, whereas the heavy fraction was less active in this respect.

The ribonucleic acid content of the light fraction was greater than that of the other two.

These results indicate that the microsome pellet as usually prepared is heterogeneous, not only so far as morphological structure is concerned, but also for the synthesis of specific proteins. The heavy microsome fraction, although morphologically heterogeneous, appears to contain the components involved in the synthesis of albumin which is destined for export from the cell.

We are greatly indebted to Dr M. A. Epstein and Dr Marian Hicks for their close collaboration on the morphological aspects of this work.

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(8) The Metabolism of [4-^{14}C]Progesterone in Humans

By R. A. Harkness and K. Fotherby. [*Clinical Endocrinology Research Unit* (*Medical Research Council*), *University of Edinburgh*]

It has been shown (Contractor & Pearlman, 1960; Chang, Slaunwhite & Sandberg, 1960) that progersterone can be metabolized to compounds more polar than pregnanediol, although the identity and quantitative significance of these metabolites have

not been definitely established. The present communication describes the pattern of metabolites found in urine from an adrenalectomized, ovariectomized woman who had received 20 μc of [4-^{14}C]progesterone intravenously.

The extract, obtained after hydrolysis of the urine with a β-glucuronidase preparation, was divided into ketonic and non-ketonic fractions (containing respectively 1·95 and 30·5% of the injected dose) which were subjected to counter-current distribution for 100 cycles using the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (7:3:5:5, by vol.). The distribution pattern of the non-ketonic fraction could be divided into three main parts. The least polar fraction, occupying tubes 39–75 of the distribution, accounted for 90% of the radioactivity of the non-ketonic fraction and contained 5β-pregnane-3α:20α-diol and its isomers. The second fraction (tubes 12–39) accounted for 5% of the radioactivity of the non-ketonic fraction and was separated by chromatography on paper and alumina into four major fractions together with at least four minor fractions. These fractions behaved chromatographically like pregnanetriols. The most polar fraction occupied tubes 0–12 and accounted for 3% of the non-ketonic fraction.

Distribution of the ketonic fraction using the above solvent system also produced three main fractions. The least polar fraction (tubes 60–84) accounted for 13·5% of the radioactivity in the ketonic fraction and contained the pregnanolones and pregnanediones. The second fraction (tubes 30–54) accounted for 28·3% of the ketonic fraction and was separated into a group of four compounds behaving like pregnanetriones and two more polar compounds. The third fraction (tubes 0–28) could be separated by further counter-current distributions into three fractions which accounted for 8·3%, 21·4% and 28·5% of the ketonic fraction. Each of these fractions contained at least three components. One component of the middle fraction behaved similarly to 3α:6α-dihydroxy-5β-pregnan-20-one.

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(9) Salt and Water Content of the Isolated Rat Heart before and after Perfusion

By J. A. Barclay, E. J. Hamley and K. White. (*Department of Physiology, University of Birmingham*)

The relative amounts of cellular sodium chloride, potassium chloride and water are known for the tissue slice to be related to the metabolic state of the tissue and the composition of the ambient solution, but the mechanisms involved in the shifts of salts and water are not yet fully elucidated. The dependence of the composition of intracellular fluid on the level of metabolic activity introduces an indeterminate factor in the interpretation of the considerable variety of analytical results obtained from a range of different tissues.

The utilization of cardiac muscle permits analyses to be obtained in circumstances where the functional integrity of the tissue is manifest. Therefore we present calculations on data from the earlier results (Barclay, Hamley & Houghton, 1960) of a continuing investigation of shifts in cellular salt and water which are consequent on perfusion. These data were obtained on intact rat hearts perfused for 2 hr. by retrograde aortic cannulation and using oxygenated Ringer–Locke solution at 30–35° and 30–33 cm. solution pressure. This method differs from the tissue-slice preparation in that this is an arterial perfusion using the coronary system of the heart.

The analytical data for such rat hearts are not dissimilar to those obtained for various tissue slices by Leaf (1956) but seem to us to require an interpretation at variance with his. Our experimental data and calculations exclude an interpretation in terms of the movement of salt and water in the proportions of isosmotic saline. The means of the values for right and left ventricles show an accumulation of sodium chloride which corresponds to the influx of a solution (1·5%) which is nearly twice the concentration of the perfusion fluid (0·9%).

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(10) Sarcoplasmic Proteins in Developing Skeletal Muscle

By D. J. Hartshorne and S. V. Perry. (*Department of Biochemistry, University of Birmingham*)

A feature of the developing muscle cell is that during foetal life an increasing proportion of the cytoplasm becomes differentiated into the specialized contractile system. As a preliminary investigation into the biochemistry underlying these processes a study in the changes occurring in the soluble sarcoplasmic proteins during foetal and early life has been undertaken. In the rabbit the sarcoplasm of adult skeletal muscle differs from the soluble proteins of liver and brain (Bondy, unpublished results) in that a relatively greater proportion of the total protein (about 80%) is eluted from diethylaminoethylcellulose at ionic strength

below 0·02. On starch-gel electrophoresis (Smithies, 1955) of whole sarcoplasm in borate buffer, pH 9·1, at least 12–14 different bands could clearly be recognized and some of these have been identified with known proteins. When whole sarcoplasm was fractionated on a column of diethylaminoethylcellulose there was a good direct correlation between the mobility of the components in each fraction and the ionic strength required for their elution from the diethylaminoethylcellulose. Whereas the majority of identifiable bands moved towards the anode, a striking feature of the electrophoretic pattern obtained was the reproducible, densely staining series of 4–5 bands which moved in the opposite direction.

In sarcoplasm isolated from the back and hind leg muscle of rabbit foetuses (26–28 days old) and from the newly born animal the components which moved towards the cathode were much reduced. The lowered content of these more basic proteins in foetal muscle was reflected by the fact that only about 15–30 % of the total protein was eluted from diethylaminoethylcellulose at ionic strength 0·02. Although the backward-moving components were very conspicuous in extracts made from the mixed back and leg muscles and from the psoas, they were less apparent in red skeletal muscle, and particularly in cardiac muscle of adult rabbits. It is of interest to speculate whether the marked increase in the backward-moving components, which is apparent on electrophoresis of white skeletal muscle after birth, is a consequence of the more highly developed anaerobic activity of this tissue compared to red skeletal and cardiac muscle.

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(11) The Incorporation of Sodium [2-^{14}C]-Acetate into Rubber by the Latex of *Hevea brasiliensis*

By R. V. Harris and R. G. O. Kekwick. (*Department of Biochemistry, The University of Birmingham*)

Bandurski & Teas (1957) found that sodium [1-^{14}C]acetate was incorporated into rubber by the latex tapped from seedling trees of *Hevea brasiliensis*. The incubation mixture contained latex diluted threefold in 0·2 M-sucrose containing added cofactors. Park & Bonner (1958), and Gascoigne & Jones (1959) were unable to obtain incorporation of labelled sodium acetate under the conditions used by Bandurski & Teas (1957). Furthermore, neither Gascoigne & Jones (1959) nor Kekwick *et al.* (1959) were able to obtain incorporation of [2-^{14}C]mevalonic acid into rubber by latex from *Hevea brasiliensis* using these conditions. However, since Park & Bonner (1958) and Kekwick *et al.* (1959) did obtain incorporation of [2-^{14}C]mevalonic acid into rubber by undiluted latex, the ability of latex to incorporate labelled acetate into rubber was rechecked.

Samples of latex were obtained from eight-year-old seedling *Hevea brasiliensis* trees grown in a hot house at Birmingham. Incubations with sodium [2-^{14}C]acetate were carried out at 28° in the absence of added cofactors, using fresh undiluted latex.

The rubber was purified and analysed by the methods previously described (Kekwick *et al.* 1959). The percentage incorporation of the radioactive carbon into the rubber obtained varied from 1·4 % with latex obtained in midsummer (August) to 0·1 % with latex obtained in midwinter (January). Duplicate incubations and analyses carried out simultaneously agreed to within 2 %. While standing in air at room temperature, the latex rapidly lost its ability to incorporate labelled acetate in the absence of added cofactors and had completely lost this ability after 2 hr.

Tapping and incubation in an atmosphere of nitrogen increased the incorporation by nearly 100 %. Tapping in air and incubation in the presence of the reducing agents cyteine (25 mM) and sodium ascorbate (22 mM) resulted in a decrease in the incorporation of labelled acetate.

Sodium [3-^{14}C]pyruvate was a less efficient precursor than sodium [2-^{14}C]acetate. The incorporation obtained was only about 50 % of that of [2-^{14}C]acetate.

Examination of the incubation mixtures for acyl-coenzyme A derivatives by chromatography of the derived hydroxamic acids revealed labelling in acetohydroxamic acid. Patrick (1957) and the present authors have found evidence for an acetate-activating enzyme in *Hevea brasiliensis* latex.

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(12) The Catabolic Rate of Albumin Doubly Labelled with ^{14}C and ^{131}I in the Isolated Perfused Rat Liver

By A. H. Gordon. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

[^{14}C]Glycine or [^{14}C]leucine was incorporated into rat plasma proteins either *in vivo* or by means of the perfused liver (Gordon & Humphrey, 1960).

After removal of fibrinogen by half-saturation with sodium chloride, the remaining proteins were iodinated with ^{131}I by the method of McFarlane (1958). Doubly labelled albumin was then separated by electrophoresis in starch gel (Gordon, 1960). In order to remove most of the soluble starch the albumin solution thus prepared was mixed with heparinized rat blood and used for a preliminary liver perfusion. After 2 hr. this liver was taken out of the perfusion circuit and replaced by another which was perfused with the same blood for a further 4 hours. The rate of catabolism of the albumin was calculated on the one hand from the amounts of $^{14}CO_2$ evolved together with the ^{14}C incorporated in the liver, and on the other from the increase in the plasma in the amount of ^{131}I soluble in 10% trichloroacetic acid.

When albumin labelled with [^{14}C]glycine and ^{131}I was used, the catabolic rate calculated from the $^{14}CO_2$ only was much greater than the rate derived from the ^{131}I-label. On the other hand, using [^{14}C]leucine the rate of $^{14}CO_2$ production was greatly reduced and the rate of catabolism calculated from the total $^{14}CO_2$ together with the ^{14}C in the liver approximated to the rate measured by means of the ^{131}I.

In similar experiments the use of whole rat plasma previously labelled *in vivo* or in a liver perfusion by means of a ^{14}C-labelled amino acid always led to high rates of evolution of $^{14}CO_2$. The present results are in accord with the view that only a small part of the albumin is normally catabolized in the liver (Cohen & Gordon, 1958). Furthermore, the different behaviour in the perfused liver of ^{14}C labelled whole plasma and [^{14}C]-leucine-labelled albumin provides an alternative explanation for the results of Green & Miller (1960) who perfused rat livers with [^{14}C]leucine-labelled whole plasma. Their view that the liver is the main site of catabolism of the plasma proteins would thus seem to be incorrect, at least for albumin.

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(13) Properties of Phosphatidic Acid Phosphatase

By R. Coleman and G. Hübscher. (*Department of Medical Biochemistry and Pharmacology, University of Birmingham*)

Much interest has centred on the role of phosphatidic acid in lipid metabolism. Hokin & Hokin (1955, 1959*a*) showed that this compound has a high rate of turnover of ^{32}P in secretory tissues and Kennedy (1957) and Paulus & Kennedy (1960) gave evidence that it is an intermediate in phospholipid and triglyceride biosynthesis. The demonstration of trace amounts of phosphatidic acid in various tissues (Hokin & Hokin, 1958) and its isolation from mammalian liver (Hübscher & Clark, 1960) has established it as a tissue constituent. Smith, Wagner, Weiss & Kennedy (1957) first described particulate preparations from chicken-liver dephosphorylating phosphatidic acid. This was confirmed by Rossiter & Strickland (1959). Hokin & Hokin (1959*b*) reported phosphatidic acid phosphatase activity in deoxycholate extracts of brain microsomes.

This investigation deals with the solubilization and further properties of phosphatidic acid phosphatase. Tissue surveys showed that phosphatidic acid phosphatase was present in the kidney, intestine, brain and liver of pig, ox, guinea pig, rat and rabbit. Pig-kidney homogenates were selected and a cell fractionation showed that 60% of the total activity was in the microsomal fraction giving a threefold increase in specific activity over the homogenate. The microsomal preparation had a pH optimum of 6·0 and a K_m of 0·2 mM.

The enzyme was unaffected by ethylenediaminetetra-acetic acid, citrate or tartrate. Ca^{2+}, Ba^{2+} and Ni^{2+} had little effect but inhibition was increased thus:

$$Mg^{2+} < Co^{2+} < Cu^{2+} < Mn^{2+} < Fe^{2+} < Zn^{2+} < Hg^{2+}.$$

Cyanide, iodoacetate or glutathione had no effect, while fluoride or *p*-chloromercuribenzoate caused inhibition. The effect of the latter compound could be reversed by glutathione. The effect of various detergents was determined (5 mg./ml.) and inhibition increased thus:

cholate < deoxycholate < Tween 20 < Teepol < dodecylsulphate < Cetavlon.

Enzymic activity remained unaltered and in particulate form after extensive freezing and thawing, high-speed blending, autolysis under toluene, or digestion with ribonuclease or wheat-germ lipase, while pancreatin partially denatured without causing solubilization. Partial solubilization was obtained with cholate and deoxycholate but recoveries were difficult to calculate owing to simultaneous inhibition.

Autolysis for 3 days followed by digestion with ribonuclease yielded particulate preparations of higher specific activity. Treatment of these with butanol caused solubilization of phosphatidic acid phosphatase without loss of activity.

These results indicate that the enzyme may be a lipoprotein.

Thanks are due to the Medical Research Council for a grant to one of us (R.C.).

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(14) Direct Esterification of Monoglycerides in Rat Intestinal Mucosa

By B. Clark and G. Hübscher. (*Department of Medical Biochemistry and Pharmacology, University of Birmingham*)

A small but significant esterification of monoglycerides has been demonstrated in liver microsomes (Stein & Shapiro, 1958; Weiss, Kennedy & Kiyasu, 1960). Reiser & Williams (1953) found that about 30% of doubly labelled monopalmitin was incorporated without hydrolysis into lymph triglycerides, indicating a direct esterification. Frazer, Pover & Sammons (1953) and Kennedy (1957) suggested that this process might be one mechanism for triglyceride re-synthesis in the intestinal mucosa.

Mitochondrial preparations carrying out the esterification of monoglycerides were isolated from rat and rabbit intestinal mucosa (Clark & Hübscher, 1960, 1961). Adenosine triphosphate and coenzyme A were required for optimal incorporation. In this communication, some properties of a particulate preparation from rat mucosa are reported. The methods used have been described previously (Clark & Hübscher, 1960).

A cell fractionation revealed that esterification of monoglycerides occurred in mitochondria, microsomes and supernatant, the relative distribution being 21, 13 and 66% of the total activity. Extraction of the mitochondrial preparation yielded a particulate sediment retaining full esterifying activity but only half of the original protein. These results suggest that the enzyme system might exist in two forms: a soluble one in the supernatant and another being part of particulate structures.

The extracted mitochondrial preparation could be stored for 3 weeks at $-10°$ without appreciable loss of activity. It had a partial requirement for coenzyme A and an absolute one for adenosine triphosphate and mono-olein. In view of this energy requirement an esterification due to lipase activity can be excluded. Optimal protein concentration was 1·5–2·5 mg./ml., higher concentrations inhibiting to varying degrees. Mono-olein (optimal concentration 3 mM) could not be replaced by α-glycerophosphate or glycerol. $MgCl_2$ (final concentration 6·7 mM) caused a 25% inhibition.

Esterification of glycerides differing in their fatty acids gave maximum activity with saturated substrates when the fatty acid chain length was between C_{10} and C_{12}. Mono-olein showed a similarly high activity. The degree of emulsification may, however, be the predominant factor in such a comparison.

Isolation of the reaction products by silicic acid chromatography (Barron & Hanahan, 1958) gave triglycerides (1·9%), diglycerides (88·8%) and monoglycerides (9·3%).

The results were discussed in relation to fat absorption.

We thank the Medical Research Council for a grant to one of us (B.C.).

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(15) Periodate Oxidation of Heparin and Related Compounds

By A. B. Foster, R. Harrison, T. D. Inch, M. Stacey and J. M. Webber. (*Department of Chemistry, The University, Birmingham* 15)

Ox-lung heparin ($[\alpha]_D + 48°$ in water; S, 13·5%; anticoagulant activity 124·5 i.u./mg.) consumed 6 mol. of periodate* during 371 hr. at 25° in the dark; oxidation was still incomplete at this stage. During this time, the amino sugar moieties were not oxidized, but the content of intact uronic acid fell by about 46% to a steady value within 60 hr. Over-oxidation of the cleaved uronic acid units probably occurred since the polysaccharide was

slowly fragmented (35% diffusible after 300 hr., initially, essentially non-diffusible) although the content of intact uronic acid in the non-diffusible fragments remained constant. The model compound, sodium methyl 4-*O*-methyl-α-D-glucopyranosiduronate (Wacek, Leitinger & Hochbahn, 1959) consumed 1 mol.prop. of periodate in 10 hr. and was then slowly over-oxidized.

Desulphation of heparin (cf. Danishefsky, Eiber & Carr, 1960) and subsequent *N*-acetylation gave a product (S, 3·3%) which consumed 2 mol. of periodate* in 5 hr., with concomitant cleavage of more than 90% of the uronic acid moieties and then was slowly over-oxidized.

The uronic acid content of the periodate-oxidized polysaccharides was determined by decarboxylation (Barker, Foster, Siddiqui & Stacey, 1958) after reduction of aldehyde groups with borohydride. Acidic hydrolysis of heparin and desulphated heparin after oxidation and reduction gave erythronic acid, thereby confirming that the amino sugar–uronic acid linkage is $1 \rightarrow 4$. Since only half of the uronic acid units in heparin were oxidized by periodate, the remainder must have been sulphated at $C_{(2)}$ or $C_{(3)}$.

De-*N*-sulphation of heparin (Foster, Martlew, Stacey, Taylor & Webber, 1961) gave a product ($[\alpha]_D + 52°$ in water; S, 8·9%) which on degradation with nitrous acid (Foster, Martlew & Stacey, 1953) gave a mixture of sulphated derivatives of D-glucuronosyl-2,5-anhydro-D-mannose isolated as the cetyltrimethylammonium salts. All the uronic acid in this mixture was cleaved by periodate, hence the $C_{(3)}$-hydroxyl groups were not sulphated and consequently in the original heparin 50% of the uronic acid moieties were sulphated at position 2. These results accord with the heparin structure postulated by Wolfrom, Montgomery, Karabinos & Rathgeb (1950).

In the deaminative degradation of heparin, the glucuronidic linkages are unaffected and $[\alpha]_D$, initially $+48°$ falls to about zero. It is difficult to reconcile this rotational change with an α-configuration for the glucuronidic linkages.

Irradiation of an oxygen-free, 0·5% aqueous solution of heparin with γ-rays at a dose rate of $1{\cdot}0 \times 10^{17}$ ev/ml./min.$^{-1}$ caused about 50% loss in anticoagulant activity without significant fragmentation of the molecule.

* Per tetrasaccharide repeating unit.

Evans Biological Institute are acknowledged for gifts of heparin.

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(16) Reaction of Heparin with $H^{14}CN$

By A. B. Foster, M. Stacey, P. J. M. Taylor, J. M. Webber and M. L. Wolfrom. (*Department of Chemistry, The University, Birmingham* 15; *Department of Chemistry, Ohio State University, Columbus* 10, *Ohio, U.S.A.*)

Identification of the reducing end-group in heparin may be of importance in determining the pattern of biosynthesis of the polysaccharide, of which little is known. Since heparin has a relatively low molecular weight of about 17 000 (Foster & Huggard, 1955), the identity of the reducing end-group has been investigated by reacting the polysaccharide with $K^{14}CN$, and degrading the adduct. The reaction of polysaccharides with $H^{14}CN$ has previously been used for molecular-weight determinations (Isbell, Frush & Moyer, 1957).

Smooth and essentially stoicheiometric reaction occurred between $K^{14}CN$ and the following series of compounds, some of which are likely reducing end-groups in heparin: D-glucurone, sodium D-glucuronate, 2-amino-2-deoxy-D-glucose hydrochloride, 2-acetamido-2-deoxy-D-glucose, and 2-deoxy-2-sulphoamino-D-glucose (Foster, Martlew, Stacey, Taylor & Webber, 1961). The $H^{14}CN$-adduct obtained from 2-amino-2-deoxy-D-glucose hydrochloride, after hydrolysis of the nitrile function, was essentially unaffected by the acid conditions necessary to degrade heparin; some loss (up to 20%) of ^{14}C-label occurred with the sodium D-glucuronate adduct.

When ox-lung heparin ($[\alpha]_D + 48°$ in water, S, 13·5%; anticoagulant activity 124·5 i.u./mg.) was allowed to react to completion with $K^{14}CN$, or was reduced with sodium borohydride, the anticoagulant activity was little affected; owing to incomplete reduction, the reduced heparin also reacted with $K^{14}CN$ (10% labelling compared with heparin). Hydrolysis of the nitrile function of the heparin $H^{14}CN$-adduct caused no appreciable change in the content of ^{14}C-label, and the extent of labelling corresponded to a molecular weight of about 14 000. Paper electrophoretic fractionation (Foster, 1952) (acetate buffer pH 5) of the acid-degraded adduct (4N-HCl, 10 hr., 100°) revealed 60% of the recovered label at the neutral point, about 20% at the position for a heptaric acid monolactone, and the remainder distributed between

these positions. The identity of the active products remains to be confirmed by carrier dilution but it seems probable that the main neutral component is 3-amino-3-deoxyheptonic acid arising from amino sugar end-groups. A second neutral product could be the dilactone of glucoheptaric acid, but, under the conditions used, the bulk of this acid is likely to be present as monolactone.

This analysis suggests that the amino sugar is the main reducing component in heparin. However, losses of ^{14}C-label on hydrolysis (about 40%) are more likely to have occurred at the expense of heptaric rather than amino heptonic acid. It should also be borne in mind that a commercial heparin preparation was employed, and that degradation may have occurred during the isolation procedure.

Evans Biological Institute are acknowledged for gifts of heparin.

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(17) Structural Dependence of Protein Ultraviolet Fluorescence

By F. W. J. Teale (introduced by S. V. Perry). (*Department of Biochemistry, University of Birmingham*)

The tyrosine component of the fluorescence of human-serum albumin (HSA) is 10% of that calculated for the free amino acid (Weber, 1961). Resonance energy transfer to tryptophan cannot be responsible for tyrosine quenching, and interaction with adjacent carboxylate groups has been suggested (Teale, 1960). Striking fluorescence changes were produced by titration of HSA with ionic detergents. The absolute quantum yield of tyrosine increased to 8%, followed by reduction of the tryptophan absolute yield to 5% from the virtually unquenched value in native HSA. In consequence the fluorescence excitation spectrum characteristic of tryptophan in HSA was progressively replaced by that of tyrosine. Similar effects were shown after titration of HSA with *N*-bromosuccinimide (Peters, 1959) to abolish tryptophan fluorescence; the residual tyrosine absolute quantum yield of 1·5% was increased to 6% by detergents. Neutral salts partly reversed the fluorescence changes, as did removal of detergent by ion-exchange. The reciprocal behaviour shown by tyrosine and tryptophan could not be related to energy transfer between these groups because the changes in yield were consecutive rather than simultaneous, and glycerol maintained the tryptophan contribution. The view that detergents alter the tertiary structure and specific quenching environment of the aromatic residues was supported by the accompanying absorption changes. Compared with native HSA in aqueous solution, the absorption decrease produced by detergents was proportional to the increase in tyrosine yield. The magnitude and sign of the absorption difference spectrum depended on the polarizability of the medium, and was zero in a medium of dielectric constant 60, indicating this value for the average environment of tyrosine groups in HSA. The intrinsic viscosity of HSA was also increased by detergents, suggesting molecular swelling by electrostatic repulsion, similar to that produced by low pH values (Tanford, Buzzell, Rands & Swanson, 1955). This mechanism agreed with the observation that anionic detergents were most effective, in view of the relatively acid isoelectric point (pH 4·6) of HSA.

The tyrosine yield increase accompanying a measured increase in molecular volume was similar to that produced by comparable concentration decrease in aqueous solutions of sodium or methyl formate. This suggested that similar short-range interactions were involved in the quenching process.

Detergents produced fluorescence changes in bovine-serum albumin, lysozyme, fibrinogen, chymotrypsin, ribonuclease and ovomucoid, comparable with those observed in HSA.

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(18) Mechanism of Squalene Biosynthesis from Mevalonate and Farnesyl Pyrophosphate

By G. J. Popják, DeW. S. Goodman, J. W. Cornforth, Rita H. Cornforth and R. Ryhage. (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London; National Heart Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.; National Institute for Medical Research, Mill Hill, London; Karolinska Institutet, Stockholm, Sweden*)

In order to study the mechanism of squalene biosynthesis from farnesyl pyrophosphate (FPP)

(Lynen, Eggerer, Henning & Kessel, 1958; Popják, 1959; Goodman & Popják, 1960) we have determined the labelling of squalene biosynthesized in liver enzyme preparations from [5-D_2-2-^{14}C]mevalonate and from [2-T_2-2-^{14}C]FPP. It was found that squalene synthesized from [5-D_2-2-^{14}C]mevalonate contained eleven out of a possible maximum of twelve atoms of D and that the labelling of the squalene with D on its two central carbon atoms was: $-CHD \cdot CD_2-$. Since C-5 of mevalonate, which furnishes the two central carbons of squalene, becomes C-1 of FPP, the results indicate that during condensation of two FPP molecules to squalene one H atom attached to C-1 of one of the two FPP molecules is exchanged. This conclusion was confirmed by using synthetic [1-T_2-2-^{14}C]FPP as substrate with the microsomal squalene synthetase system (Goodman & Popják, 1960). Taking the ratio of T/^{14}C in the [1-T_2-2-^{14}C]FPP to have been 1·00, the ratio in the squalene synthesized from it was $< 0{\cdot}77$ indicating the loss of one labelled H atom out of four.

By the use of T-labelled reduced triphosphopyridine nucleotide (TPNH) and of THO (tritium-labelled water) it was shown that the origin of the one H atom attached to one of the central carbons of squalene, and which is not derived from the hydrogens attached to C-1 of FPP, is the reduced pyridine nucleotide and not the water of the incubation medium. There was no incorporation of a proton from the water of the incubation medium into squalene when this was synthesized from FPP with the microsomal squalene synthetase system.

The observations are interpreted to mean that an asymmetric process is involved in the synthesis of the symmetrical molecule of squalene in the sense that one of the two FPP molecules condensing is subject to reactions different from those of the other. Also the role of TPNH in squalene biosynthesis cannot be the reduction of a double bond, for in that event not only a hydride ion (from TPNH) but also a proton from the water should have entered the squalene molecule. Possible reaction mechanisms were discussed.

The finding that chemically synthesized *trans-trans*[1-T_2-2-^{14}C]FPP (and also *trans-trans*[1-D_2-2-^{14}C]FPP) acted as substrate for squalene synthesis, provided also a formal proof for the identity of the immediate precursor of squalene deduced previously from the study of trace (or μmole) amounts of biosynthetic material (Lynen *et al.* 1958; Goodman & Popják, 1960).

The results obtained are at variance with those of Rilling & Bloch (1959) on the same subject.

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(19) The Metabolism of 2-Deoxy-2-Fluoroglyceraldehyde

By D. H. Treble* and R. A. Peters. (*Department of Biochemistry, University of Cambridge*)

O'Brien & Peters (1958) showed that 2-deoxy-2-fluoroglyceraldehyde (FGA) was toxic to mammalian systems causing citrate to accumulate. They proposed that FGA is metabolized to fluorocitrate which strongly inhibits aconitase, thereby causing the accumulation of citrate (Peters, 1952).

This work has been extended and a pathway proposed for the conversion of FGA into fluorocitrate.

Four enzymes, capable of oxidizing aldehydes, were tried on FGA. Liver aldehyde oxidase (Mahler, Mackler, Green & Bock, 1954) had no effect, phosphoglyceraldehyde dehydrogenase a very slight one. Xanthine oxidase oxidized FGA only 20% as fast as acetaldehyde but liver aldehyde dehydrogenase oxidized FGA twice as fast as acetaldehyde and about 40% of the rate of DL-glyceraldehyde. The oxidation of FGA to 2-deoxy-2-fluoroglycerate could therefore occur at a significant rate *in vivo*.

2-Deoxy-2-fluoroglycerate causes more citrate to accumulate than FGA does in kidney mitochondria used as described by O'Brien & Peters (1958), and could therefore be intermediate between FGA and fluorocitrate. Decarboxylation to 2-fluoroethanol or oxidation to fluoromalonate were ruled out since these possible intermediates are not toxic under conditions where 2-deoxy-2-fluoroglycerate is toxic (unpublished observations). Serine hydroxymethylase (Blakley, 1955) converts serine into glycine. If 2-deoxy-2-fluoroglycerate behaves as a structural analogue of serine then this enzyme would form fluoroacetate directly. That it does so is suggested by the fact that (−)-2-deoxy-2-fluoroglycerate is much more toxic to kidney mitochondria than its stereoisomer. Comparison with the closely related glycerates suggest that the toxic isomer is probably L in configuration (Kent, Hebblethwaite & Taylor, 1960). Incubation of (±)-2-deoxy-2-fluoroglycerate with partially purified enzyme resulted in the formation of formaldehyde, as determined with chromotropic acid by the method of O'Dea & Gibbons (1953) compared with an enzyme-free control.

* Wellcome Research Fellow.

Thus FGA can be metabolized to bring about the lethal synthesis of fluorocitrate which inhibits aconitase and causes death by the action in turn of four enzymes, aldehyde dehydrogenase, serine hydroxymethylase, acetate-activating enzyme and condensing enzyme.

We are grateful to Dr P. W. Kent for the gift of 2-deoxy-2-fluoroglyceraldehyde and 2-deoxy-2-fluoroglycerates, and to Dr B. C. Saunders and Dr D. E. Bronert for 2-fluoroethanol and fluoromalonate, and to Professor F. Bergel and Dr R. C. Bray and Dr D. A. Gilbert for the gift of pure xanthine oxidase. We wish to thank Mrs M. A. Davies for skilled assistance, and the Wellcome Trust and Shell Petroleum Co. Ltd for financial support.

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(20) The Inability of Vitamin A Acid to Replace Vitamin A in Pregnant Female Rats

By J. N. Thompson, J. McC. Howell and G. A. J. Pitt. (*Departments of Biochemistry and Veterinary Pathology, University of Liverpool*)

Vitamin A acid, the carboxylic acid corresponding to the primary alcohol, vitamin A, has previously been found to replace vitamin A alcohol in all functions of vitamin A investigated *in vivo*, except that it cannot provide vitamin A aldehyde for the prosthetic group of the visual pigments. It has been suggested that vitamin A acid is the form of the vitamin active systemically, i.e. concerned with growth and general tissue maintenance (Moore, 1953; Dowling & Wald, 1960).

We now report that vitamin A acid is ineffective in maintaining reproduction in female rats.

Female rats were reared from weaning on a diet deficient in vitamin A alcohol but supplemented with vitamin A acid. When mated with normal males they became pregnant but the foetuses were resorbed and no litters were born. If the females were given vitamin A alcohol (as the acetate) shortly before mating, they bore normal litters.

The same results were obtained whether the vitamin A acid was given as twice-weekly doses or mixed with the food to provide a continuous supply. Doses varying from 100 μg. of vitamin A acid/day to about 3 mg./day (i.e. up to the hypervitaminotic level) were all ineffective.

The foetal sites have been studied from animals given a vitamin A-deficient diet supplemented with vitamin A acid at a low and a high level. At the end of the second week of pregnancy, areas of polymorph infiltration and necrosis were seen in the placentae, similar to the lesions observed in vitamin A deficiency (Mason, 1935). No lesions were seen in the foetuses.

The placenta therefore seems to differ from other tissues of the body and to resemble the rod layer of the retina (Dowling & Wald, 1960) in not being adequately maintained by vitamin A acid.

We conclude that the role of vitamin A in supporting reproduction in females is different from its 'systemic' mode of action in growth and general tissue maintenance, since the needs of the latter are met by vitamin A acid. It seems probable that vitamin A works in reproduction as the alcohol or aldehyde.

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DEMONSTRATION

A Collapsible Metabolic Cage

By R. F. Denman (introduced by Elizabeth Work). (*Twyford Laboratories, Twyford Abbey Road, London, N.W.* 10)

The cage was designed for metabolic experiments involving radioactively labelled compounds, and so is made from materials which would not be scratched by the animal. It is constructed of stainless steel and glass. Four glass sides are held in place by two identical steel wire units that are looped through the grid floor. A funnel made from a cut polythene bag is suspended underneath, so directed that the excreta fall on to a modified form of the conventional 'Hopkins' type separator. After use, the cage may be dismantled for cleaning and storage, and the funnel is incinerated. The cage may be used for all normal purposes. The wall materials may be changed or modified according to experimental requirements.

[*The Editors of* The Biochemical Journal *accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **406th Meeting** *of the Society was held in University College and Trinity College, Dublin, on Thursday and Friday, 25 and 26 May* 1961, *when the following papers were presented:*

COMMUNICATIONS

(1) The Trehalose–Glycogen Relationship in Baker's Yeast

By E. R. Tully, R. F. Palmer and T. G. Brady. (*Department of Biochemistry, University College, Cork*)

In an attempt to explain the variation in results obtained when inducers of endogenous fermentation were added to different yeast samples, a study was made of the carbohydrate reserves of yeast under varying experimental conditions.

The four sugar polymers, glycogen, trehalose, mannan and glucan, were fractionated by the Trevelyan & Harrison (1956) procedure and the carbohydrate content of each fraction estimated by the modified anthrone method of Chen (1959).

As anticipated, the glucan and mannan fractions showed little fluctuation under the conditions employed. Both glycogen and trehalose levels altered appreciably, however, and under certain conditions in an inverse manner, indicating an interrelationship between the metabolic pathways of these two substances. A possible interpretation of this interrelationship was proposed.

Chen, S. L. (1959). *Biochim. biophys. Acta*, **32**, 470.
Trevelyan, W. E. & Harrison, J. S. (1956). *Biochem. J.* **63**, 23.

(2) Survey of the Distribution and Nature of Mammalian Adenosine Deaminases

By T. G. Brady and Miss C. I. O'Donovan. (*Department of Biochemistry, University College, Cork*)

Adenosine deaminase activity has been found in all of the organs of the rat, guinea pig, rabbit, cat and dog that have been tested. The highest activity in most species was present in the spleen and high activity was also present in the alimentary tract. The exceptionally high deaminase activity in cat lung, first observed by Clarke, Davoll, Phillips & Brown (1952) was confirmed. Lung tissue from other species had only moderate activity.

Enzyme activity was measured using adenosine and deoxyadenosine as substrates. Both were deaminated by all the tissues examined. However, the tissues of the rabbit and dog deaminated adenosine at a faster rate than deoxyadenosine, while the tissues of the rat, guinea pig and cat deaminated both substrates at almost equal rates.

Clarke, D. A., Davoll, J., Phillips, F. S. & Brown, G. B. (1952). *J. Pharmacol. exp. Therap.* **106**, 291.

(3) Influence of pH and Urea on the Activity of Mammalian Adenosine Deaminase

By T. G. Brady and M. O'Sullivan. (*Department of Biochemistry, University College, Cork*)

The effect of pH on purified adenosine deaminase in the range pH 3·5–11·0 was tested for periods up to 24 hr. at 37°. The enzyme showed no fall in activity between pH 5 and 9, but was rapidly inactivated below pH 4 and above pH 9·5.

Urea concentrations of 6M or lower did not cause inactivation of the enzyme. The inactivation caused by 8M-urea was both pH- and temperature-dependent and in each case passed through a minimum.

Spectrophotometric titration of the enzyme at 295 mμ in the presence and absence of urea showed no significant difference, indicating that hydrogen bonding of tyrosine residues does not occur in this enzyme (Stracher, 1960).

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(4) Studies on the Mechanism Controlling Glutamyl-Transferase Activity in Mammalian Cells

By P. Fottrell* and J. Paul. (*Department of Biochemistry, University of Glasgow*)

The repression of glutamyl-transferase activity in HeLa cells grown in the presence of glutamine (De Mars, 1958) indicates that animal cells in common with bacteria (Yates & Pardee, 1957; Vogel, 1957; Magasanik & Karibian, 1960) possess mechanisms for the regulation of certain enzymic pathways. We have found that the activity of glutamyl transferase (which is almost

* Beit Memorial Fellow.

certainly identical with glutamine synthetase) in mouse subcutaneous fibroblasts, strain L, clone 929 (Sanford, Earle & Likely, 1948) and in human-liver epithelial cells, strain HLM (Leslie, Fulton & Sinclair, 1956) increases eight- to ten-fold if the cells are grown in a medium lacking glutamine and these systems have been used in this study to investigate the mechanism of the phenomenon.

The cells were grown as monolayers on glass in Waymouth's (1959) medium, supplemented with 5% dialysed calf serum using standard techniques (Paul, 1960). Substitution of 20 mM-glutamic acid for glutamine in the culture medium resulted in increased glutamyl-transferase activity, maximum activity being reached after 48 hr. If glutamine was reintroduced into the medium the transferase activity progressively decreased until the initial level was reached after 12 hr. When attempts were made to induce the enzyme in a medium containing 0·2 mM-*p*-fluorophenylalanine or 0·2 mM-8-azaguanine as inhibitors of protein and ribonucleic acid synthesis respectively, no increase in transferase activity was detected. In contrast to these observations the inclusion of 2 μM-fluorodeoxyuridine in the medium, to inhibit deoxyribonucleic acid synthesis, had no effect on the induction of glutamyl transferase.

The repression by glutamine of the transferase activity of 'induced' cells occurs too quickly to be explained by dilution of the enzyme during growth of the cells. Studies on a crude enzyme preparation revealed that transferase activity progressively diminished during incubation at 37°. However, the enzyme could be stabilized by various factors, including nitrogen, glutamic acid, glutamine, reduced diphosphopyridine nucleotide and ascorbic acid. On the other hand, iodoacetic acid inhibited transferase activity. It is concluded from these results that sulphydryl groups at or near the active site of the enzyme are necessary for glutamyl transferase activity and whereas enzyme induction requires active protein and ribonucleic acid synthesis, inactivation may occur by oxidation of sulphydryl groups, accompanied by repression of further enzyme synthesis.

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(5) The Intracellular Distribution, Latency and Electrophoretic Mobility of L-Glutamate–Oxaloacetate Transaminase of Rat Liver

By J. W. Boyd (introduced by G. D. Greville). (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

A two- or three-fold activation of L-glutamate–oxaloacetate transaminase (GOT) in isotonic liver homogenates was achieved by various treatments, including mechanical disintegration in a Waring Blendor, ultrasonic disintegration and hypotonicity. It was shown by ultrasonic disintegration of subcellular fractions that the latent GOT was localized in the mitochondrial fraction. Previous investigators (Green, Leloir & Nocito, 1945; Fleisher, Potter, Wakim, Pankow & Osborne, 1960) have separated GOT into different fractions by means of electrophoresis. In this study the GOT of an extract of rat liver was separated into two peaks on paper electrophoresis. It was also shown that GOT in a mitochondrial extract and in the final supernatant from fractionation each has one major peak corresponding to one of the peaks of whole liver extract. The mitochondrial and supernatant enzymes were shown to differ in their substrate affinities and pH dependence as well as in their electrophoretic mobility.

These experiments were carried out using the assay method of Karmen (1955). L-Aspartate and α-oxoglutarate are substrates and oxaloacetate formed is reduced by added malic dehydrogenase and reduced diphosphopyridine nucleotide (DPNH). The oxidation of DPNH is followed in a spectrophotometer. Other assay methods (Reitman & Frankel, 1957; Cammarata & Cohen, 1951; Müller & Leuthardt, 1950) failed to give linear progress curves at the maximum rate, particularly with mitochondrial GOT. Moreover, in the assay system of Cammarata & Cohen (1951), in which oxaloacetate accumulating is measured spectrophotometrically, it was demonstrated that the affinity for α-oxoglutarate was lower than in the Karmen (1955) method. This was most marked with mitochondrial GOT. These findings indicated that the oxaloacetate formed was acting as a partial competitive inhibitor. Further evidence in support of this was obtained with the method of Müller & Leuthardt (1950) in which the other product of the reaction, L-glutamate, is determined. It was found that if malic dehydrogenase and DPNH were added to reduce the oxaloacetate formed, then glutamate formation was accelerated to equal the rate of oxaloacetate formation as determined by the Karmen (1955) method.

It is concluded that, owing to its latency and inhibition by oxaloacetate, the maximum level of

activity of GOT in mitochondria has been underestimated.

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(6) Cerebral Adenosine Triphosphatases Differing in Cellular Location and Cation Activation

By D. H. Deul and H. McIlwain. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

A fraction of ground rat brain sedimented at $10^6 g$ min. was found by Järnefelt (1961) to liberate phosphate from adenosine triphosphate at about 60 μmoles/hr. with material from 1 g. of tissue, in the presence of Mg^{2+} and K^+ salts. The liberation was accelerated about 30 % by Na^+ salts, and the significance of this in relation to the observations of Skou (1957, 1960) and Post, Merritt, Kinsolving & Albright (1960) on adenosine triphosphatases in cation transport was indicated. Hess & Pope (1957) had, however, already observed 60–100 % increase in the adenosine triphosphatase of unfractionated brain suspension by Na^+, and fractions more sensitive to Na^+ thus appeared likely to exist. In guinea-pig cerebral cortex reassimilation of K^+ can proceed at 500 μequiv./g./hr. and is inhibited by protamine and restored by ganglioside preparations (McIlwain, Woodman & Cummins, 1961); the fractions of the tissue richest in gangliosides and most active in combining with basic proteins are sedimented at 3 to $10 \times 10^6 g$ min. (Wolfe & McIlwain, 1961; Wolfe, 1961).

The properties of a microsomal fraction, Ms, separated from guinea-pig cerebral cortex at $10^7 g$ min. have now been compared with those of other fractions. The cortex, passed through a 25 μ orifice (Emanuel & Chaikoff, 1957), was differentially centrifuged giving nuclei and myelin, at $8000 g$ min.; Mt, mitochondria and small nuclei, at $50\,000 g$ min.; MtMs, mitochondria and microsomes, at $200\,000 g$ min.; Ms, and a supernatant. Phosphate liberation by Ms, from 3 mM-Mg^{2+} adenosine triphosphate at 37° usually in 2-amino-2-hydroxymethylpropane-1:3-diol buffer (pH 7·4) with 30–120 mM-KCl, was very markedly accelerated by NaCl, to values 250 % of those found in its absence. Liberation by Mt was accelerated 0–30 % by Na^+, according to the K^+ present; MtMs was intermediate in properties.

The unfractionated suspension liberated about 2000 μmoles of phosphate/g. of cortex/hr. with Mg^{2+}, K^+ and Na^+ salts and over 90 % of this was accounted for in the fractions, Mt giving 350, MtMs 490, and Ms 590 μmoles/hr. with material from 1 g. In pH optima the fractions differed markedly, Mt being at 9·1 and Ms at 7·4. Activities of Mt and Ms were little affected by simple anions but inhibited by suramin, and by ganglioside preparations (0·1–0·3 mg./ml.) which proved more selective towards Ms. Speed and sensitivity of reaction as now observed give added support to the likely involvement of the Ms adenosine triphosphatase in cation transport (McIlwain, 1961*a*, *b*).

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(7) Sialic Acid Release by Rennin Action on Cow and Human Milk

By F. H. Malpress. (*Department of Biochemistry, Queen's University, Belfast*)

When rennin acts on casein, forming paracasein, glycopeptide fractions containing neuraminic or sialic acids are liberated (Brunner & Thompson, 1959; Nitschmann & Henzi, 1959). Some quantitative aspects of this change are reported. Casein precipitated at pH 4·6 from unpasteurized skimmed cow's milk had a sialic acid content of 0·21 %, of which a maximum amount of 68 % was released by the action of crystalline rennin (pH 6·8;.25°). This result was obtained by measuring either the fall in sialic acid content of the casein after rennin action or the increase in sialic acid in the supernatant fluid after removing protein in 12 % trichloroacetic acid. Similar values were obtained when rennin clotted milk directly.

A sample of bulked human milks collected from the fourth to the eighth day of lactation showed no

precipitation at the isoelectric point. After the action of rennin under the above conditions, however, precipitation of paracasein containing 1·1% of sialic acid occurred at the isoelectric point, pH 4·7 (cf. 0·07% for paracasein from cow's milk). Estimation of the sialic acid released by rennin action gave a maximum derived figure for the sialic acid content of human-milk casein of 2·1%, of which not more than 50% can be liberated by the enzyme.

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Nitschmann, H. & Henzi, R. (1959). *Helv. chim. acta*, **42**, 1985.

(8) Levels of Some Metabolites in Normal, in Iron-deficient, and in Zinc-deficient *Mycobacterium smegmatis*

By F. G. Winder and C. O'Hara. (*Medical Research Council of Ireland Laboratories, and Department of Biochemistry, Trinity College, Dublin*)

Normal *Mycobacterium smegmatis* was grown on Proskauer and Beck medium containing 2 μg. of iron/ml. and 0·4 μg. of zinc/ml. (Winder & Denneny, 1959). Exhaustion of nitrogen and carbon sources led to progressive inhibition of growth after about 3 days. In deficient cultures the level of one of the above metals was reduced to 3% of normal, resulting in partial inhibition of growth after about 2·3 days, when the yield of bacteria was 10% of maximal. Flasks of each type of culture were harvested after 2–5 days growth, and the metabolites discussed below were extracted from the washed bacteria, determined, and their levels calculated in terms of cold-acid-insoluble bacterial nitrogen.

The bacteria became elongated in iron deficiency and to a lesser extent in zinc deficiency, while they decreased substantially in length on carbon or nitrogen exhaustion.

Ribonucleic acid level was reduced in bacteria deficient in either metal, apparently before growth was inhibited, but base composition of this nucleic acid was normal. Deoxyribonucleic acid level also was reduced before growth inhibition in both deficient bacteria. By contrast, the level of this nucleic acid increased substantially when either carbon or nitrogen became exhausted.

In zinc deficiency the insoluble inorganic polyphosphate level increased several times, indicating that energy- and phosphate-using reactions were inhibited more than oxidative phosphorylation. In iron deficiency the increase stopped after a short time, showing that oxidative phosphorylation also was inhibited. Adenosine triphosphate level dropped in normal and in iron-deficient cultures when growth slowed, suggesting inhibition of adenylic acid synthesis, while in zinc-deficient cultures this level remained high. The level of pyridine nucleotides fell before growth inhibition in iron deficiency and rather later in zinc deficiency.

Coproporphyrin level normally rose to a very variable peak at 3 days and then fell with age. Iron-deficient bacteria had lower, and zinc-deficient bacteria usually higher, levels than normal at 3 days. Levels in these cultures rose in later stages of the deficiency.

In normal cultures the total lipid level was halved between 2 and 5 days, while phospholipid remained constant. In neither deficiency condition was there a comparable fall in total lipid, while phospholipid rose.

Protein per unit dry weight fell slightly in zinc deficiency.

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(9) Tryptophan Synthetase in *Mycobacterium smegmatis*

By C. Ratledge and F. G. Winder. (*Medical Research Council of Ireland Laboratories, and Department of Biochemistry, Trinity College, Dublin*)

Extracts were prepared by grinding freeze-dried *Mycobacterium smegmatis* suspended in potassium phosphate buffer, pH 7·2, with Ballotini beads in a chilled mortar for 4–5 min., and centrifuging at about 2000*g* for 15 min. at 1°. Tryptophan synthetase was determined in the supernatants, within a few hours of preparation, using the method of Yanofsky (1955) following both indole disappearance and tryptophan formation.

The synthesis of tryptophan from indole was entirely dependent on the presence of serine (80 mM) and pyridoxal phosphate (80 μM), but did not require reduced glutathione. Tryptophan formed was equivalent to the amount of indole which disappeared, the rate of reaction being linearly proportional to the amount of enzyme added up to 3·2 units. The enzyme was 50–70% inactivated on shaking extracts in a Mickle disintegrator for 90 sec. in the presence of air.

Extracts from bacteria grown for 3 days on iron-deficient medium (Winder & O'Hara, 1961) had a slightly higher specific activity of tryptophan synthetase (about 0·78 unit/mg. of protein) than extracts from bacteria grown for the same time on full medium or zinc-deficient medium (both about

0·53 unit/mg. protein). The specific activity of tryptophan synthetase in extracts from both normal and iron-deficient cells remained fairly constant from the third to the fifth day of growth of the cells. However, the specific activity of the extracts from zinc-deficient cells declined, until after 5 days growth the extracts exhibited only half the activity of those obtained from normally grown cells.

The addition of zinc (1–100 μM), iron (1–10 000 μM), or magnesium (100 μM) to the assay mixture did not restore the loss of activity in the extracts from zinc-deficient cells and did not affect the activity of the other extracts. Zinc at 10 mM produced a 30% inhibition with all extracts. L-Tryptophan (0·2 mM) was not attacked when it replaced indole (0·4 mM) in the assay mixture. This was also true when serine was omitted or when either iron or zinc (0·1 mM) was added. Mixing of extracts from normal and from metal-deficient cells did not produce any synergistic or antagonistic effects as indicated by indole consumed or tryptophan formed.

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(10) Levels of Some Enzymes in Normal, in Iron-Deficient, and in Zinc-Deficient *Mycobacterium smegmatis*

By F. G. Winder, C. O'Hara and C. Ratledge. (*Medical Research Council of Ireland Laboratories, and Department of Biochemistry, Trinity College, Dublin*)

Normal, iron-deficient, and zinc-deficient cultures of *Mycobacterium smegmatis* were harvested after 2–5 days growth (Winder & O'Hara, 1961) and the bacteria washed and smashed by shaking at 4° with Ballotini beads and appropriate buffer in a Mickle disintegrator. Enzyme activities were determined in the supernatants after centrifuging at 2000*g* for 10 min. at 4°, and the specific activities were calculated in terms of protein.

In normal cultures the glycerol-dehydrogenase activity (Burton, 1955) (about 0·02 unit/mg. of protein) altered little with age. In iron-deficiency the activity fell to about a half after 3 days growth, and in zinc-deficiency to about a third after 3 days, and lower subsequently.

In normal cultures the lactic-dehydrogenase activity (Appleby & Morton, 1954) (about 0·6 μmole ferricyanide reduced/hr./mg. of protein) also was little affected by age. In zinc-deficiency it fell to about a quarter after 4 days. In iron-deficiency a slight fall was observed.

Succinic-dehydrogenase activity (Bonner, 1955) caused a decrease in extinction of about 0·01/min./mg. of protein at 3 days in normal cultures, and about a quarter of this in iron-deficient cultures. The activity in zinc-deficient cultures fell only later.

In iron-deficient cultures catalase and peroxidase activities (Winder, 1960) were about a third of normal after 3 days, but did not fall further, whereas in zinc-deficient cultures the decrease in activity of these enzymes occurred later.

Asparaginase activity (Meister, 1955) (about 0·15 unit/mg. of protein) was not significantly affected by age of the culture or by a deficiency of either metal. Glutamic–pyruvic transaminase (Reitman & Frankel, 1957) (about 8 units/mg. of protein) was slightly increased by iron deficiency and decreased by zinc deficiency.

The following enzymes were not detected in the extracts: guanine nucleosidase, uracil oxidase, cytidine deaminase, diphosphopyridine nucleotidase, diphosphopyridine nucleotide pyrophosphorylase.

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(11) Lipid Peroxides in *Mycobacterium tuberculosis*

By J. J. Doherty and F. G. Winder. (*Medical Research Council of Ireland Laboratories, and Department of Biochemistry, Trinity College, Dublin*)

Unsaturated lipids autoxidize to form hydroperoxides. The presence of such lipids in mycobacteria,

and the report that lipid peroxides are formed during exposure of mycobacteria to cerase (Muftik, 1951), prompted us to examine whether detectable levels of these peroxides existed in living mycobacteria.

Mycobacterium tuberculosis BCG was grown on Proskauer and Beck medium (Winder & Denneny, 1956). Samples (0·25–0·75 g. wet wt.) were washed with water at 5–10° and extracted at 0–4° in diffuse daylight with three changes of each of the following solvents: ethanol (redistilled after refluxing with NaOH and Al), acetone (redistilled after treatment with NaOH), ethanol–peroxide-free ether (3:1, v/v), and benzene (redistilled after treatment with H_2SO_4). Further treatment with hot solvents yielded lipid lacking in detectable peroxide. Total nitrogen in the residues was determined. Peroxides were estimated in the extracts by a Fe^{2+} oxidation method (Koch, Stern & Ferrari, 1958). This method is not specific for peroxides, but the reacting substance(s) present in extracts of mycobacteria were destroyed by cytochrome *c* in a fashion similar to true peroxides.

Freshly prepared solvents are important for reliable results. The assay gave high results in light with acetone present, particularly samples of acetone which gave a high blank. The plot of $\sqrt{E_{480\,m\mu}}$ against concentration of acetone in the assay was approximately linear under constant light conditions and could be used to correct the results, but the use of acetone is better avoided.

Isoniazid-sensitive BCG aged 21–40 days gave 5–20 m-moles of lipid peroxide/mole of N_2 by this method. The level of peroxide was not affected by shaking the flasks of bacteria aerobically at 37° in the dark for several days before sampling.

Isoniazid-resistant BCG, lacking catalase and peroxidase, contained a similar peroxide level. Hence it would seem that these enzymes do not affect the formation of lipid peroxides in living mycobacteria.

Sensitive and resistant strains were shaken for several days in the presence and absence of 10 μg./ml. of isoniazid. Isoniazid produced no alteration in the lipid peroxide level in either strain. This finding provides no support for the suggestion that isoniazid action involves free-radical formation (Winder, 1960).

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(12) Changes in the Cytochrome of Brewer's Yeast *(Saccharomyces cerevisiae)*

By J. P. Hennessy (introduced by A. K. Mills). [*Chemist's Laboratory, Arthur Guinness Son and Co. (Dublin) Ltd., Dublin*]

Ephrussi & Slonimski (1950) and Slonimski (1953) found an absorption band at 580 mμ in anaerobic yeast and in their 'petite colony' mutants which were deficient in cytochromes *b* and *a* and a_3. Keilin (1953) and Keilin & Tissieres (1954) reported the presence of haemoglobin in samples of baker's and brewer's yeast showing an absorption band at 583 mμ on oxidation and not under reducing conditions. Ycas (1956) found that antimycin 'A' added to yeast growing aerobically stimulated the development of a compound showing an absorption band at 583 mμ under oxidizing conditions. Some observations made using Guinness brewer's yeast suggest a connexion between the compound responsible for absorption at 583 mμ and the normal cytochromes of brewer's yeast.

Brewer's yeast grown anaerobically, and showing absorption bands for cytochromes a_1 at 590 mμ and b_1 at 558 and 532 mμ showed a change in spectrum when incubated overnight at 37°. The bands for cytochromes a_1 and b_1 disappeared and two new absorption bands appeared at 583 mμ and at 549 mμ. Both were intense bands easily seen at room temperature. The yeast after the incubation had liquefied and was not viable. On centrifuging at pH 8 the clear supernatant contained both compounds. There is no evidence yet to decide whether the changes produced were enzymic or purely chemical. The effect did not take place with samples of baker's (aerobic) yeast treated similarly.

The production of two compounds absorbing at 583 and 549 mμ respectively, coincident with the disappearance of cytochromes a_1 and b_1, is of interest in hypotheses of the biosynthesis of cytochromes *a*, a_3, *b* and *c* from cytochromes a_1 and b_1. The occasional appearance of a band at 583 mμ in normal brewer's yeast and usually in respiratory mutants together with the similarity between the second absorption band at 549/550 mμ and the one normally shown by cytochrome *c* suggests as a working hypothesis the following biosynthetic pathways from cytochrome b_1:

(i) cytochrome $b_1 \rightarrow$ compound 549 m$\mu \rightarrow$ cytochrome *c*,

(ii) cytochrome $b_1 \rightarrow$ compound 583 m$\mu \rightarrow$ cytochrome $a_1 \rightarrow$ cytochromes *a* and a_3,

(iii) cytochrome $b_1 \rightarrow$ cytochrome *b*.

It is conceivable that a mutation could cause an enzymic block in (ii) leading primarily to accumulation of compound 583 mμ and absence of cytochromes a_1, *a* and a_3, with consequent decrease in

electron transfer and absence of cytochrome *b* as a secondary effect.

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(13) Insulin and the Membrane Potential of Frog Sartorius Muscle

By R. P. Kernan (introduced by E. J. Conway). (*Department of Biochemistry, University College, Dublin*)

Zierler (1957) found that freshly dissected rat muscles immersed in Ringer fluid containing insulin accumulated potassium and had a higher membrane potential than their companion muscles immersed in Ringer without insulin. He stated that the increase in membrane potential observed was in excess of that predictable from the potassium movement into the fibres, and concluded that the potassium influx was a result of hyperpolarization of the muscle-fibre membrane.

Here the resting potential of frog sartorius muscle was measured in Ringer–Conway fluid, having the same inorganic composition as frog plasma (Boyle & Conway, 1941). The microelectrode technique (Graham & Gerard, 1946) was used. The muscles were then analysed for potassium, and the potassium concentration in the fibre water calculated. The membrane potential was then calculated by the Nernst equation,

$$E_m = \boldsymbol{R}T/F \ln [K_1]/[K_0],$$

where $[K_1]$ and $[K_0]$ are the potassium concentrations in the fibre water and Ringer fluid respectively.

The mean potential found with the microelectrode technique was 91·6 mv. The calculated potential was 100·0 mv. The companion muscles were soaked in Ringer containing insulin (50 units/l.) and the mean membrane potential measured. These muscles were also analysed and the potential calculated as above. Here the observed potential was 99·4 mv and the calculated potential 100·4 mv.

Experiments were also carried out using muscles made rich in sodium and low in potassium by overnight soaking in cold potassium-free Ringer fluid. The mean potential of one set of muscles was measured soon after immersion in insulin–Ringer fluid. The potential of their companion muscles was measured after 4 hr. immersion in this fluid. Analysis of both sets of muscles showed that the potassium increased from 91 to 129 mM/l. of fibre water over this period. The potentials corresponding to these potassium levels were calculated. For the sodium-rich muscles the mean observed potential was 94 mv, that calculated being 92 mv. After recovery the observed potential was 98·6 mv and the calculated potential 98·8 mv.

It has been observed (Adrian, 1956; Kernan & Conway, 1955) that the resting potential of muscle in normal Ringer fluid is always less than that calculated from the Nernst equation, the difference being due perhaps to entry of sodium into the muscle fibres. Insulin added to Ringer fluid brings about better agreement between observed and calculated potentials rather than a hyperpolarization of the fibre membrane leading to potassium entrance.

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(14) Anion-Exchange in Frog Sartorius Muscle

By W. McD. Armstrong (introduced by E. J. Conway). (*Department of Biochemistry, University College, Dublin*)

Previous studies in this laboratory (Carey, Conway & Kernan, 1959) have revealed interesting differences in the efflux of $^{24}Na^+$ from frog sartorius muscles labelled *in vivo* by injection of the isotope, and Na^+-rich muscles prepared by overnight immersion at 0° in K^+-free Ringer solution containing $^{24}Na^+$. Anion-exchange under similar conditions has now been investigated using $^{82}Br^-$ as the tracer ion. Preliminary experiments had indicated that $^{82}Br^-$ did not differ significantly from $^{36}Cl^-$ either in its distribution in the muscle or in its rate of exchange with external Cl^- (cf. Harris, 1958).

Freshly excised sartorii from frogs, injected overnight with small amounts of $Na^{82}Br$, were suspended in inactive Conway–Ringer solution (Boyle & Conway, 1941) containing 104 m-equiv. Na^+ and 10 m-equiv. K^+/l. and the wash-out curve for $^{82}Br^-$ determined using the flow technique of Keynes (1954). The curve obtained at 19° showed the presence of a faster- and slower-moving component which may be tentatively identified with extracellular and intracellular $^{82}Br^-$ (Levi & Ussing, 1948; Harris, 1958). Emergence of the faster component appeared to be complete in about 10 min. but the zone of transition between the two parts of the curve was rather indefinite so that it

was not feasible to calculate a representative rate constant for the faster component. A mean rate constant of 4·1 hr.$^{-1}$ was found for the slower component. Similar experiments at 5° gave a mean rate constant of 2·9 hr.$^{-1}$ for this component. These values gave a Q_{10} of 1·26 for the slower-moving component, a figure strongly suggestive of a passive diffusion process.

The efflux of $^{82}Br^-$ from Na^+-rich muscles prepared by loading overnight at 0° in K^+-free Ringer solution containing 120 m-equiv. of Na^+/l. and subsequently washed out in Conway–Ringer solution (104 m-equiv. Na^+ and 10 m-equiv. K^+/l.) was also investigated. The mean rate constants obtained for the slower moving isotope fraction in these experiments were 4·6 hr.$^{-1}$ at 20° and 2·8 hr.$^{-1}$ at 5°, the Q_{10} being 1·39. These results indicate that the exchange of $^{82}Br^-$ (and, presumably, of internal Cl^-) with external Cl^- is a passive process, both *in vivo* and under conditions where Na^+ is being actively extruded from the muscles.

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(15) Effect of Increasing External Potassium on the Uptake of Oxygen in the Isolated Sartorius of the Frog

By Marie Mullaney (introduced by M. G. Harrington). (*Department of Biochemistry, University College, Dublin*)

Groups of fresh sartorii were immersed in phosphate-buffered Ringer solution in which the potassium content varied from 0 to 30 m-equiv./l. During the first hour of the immersion the potassium increase from zero to 30 m-equiv. had very little effect on the oxygen uptake. During the second hour there was a marked decrease in the oxygen consumption up to 10 m-equiv. of external potassium. This was followed by a steep rise which brought the level of the oxygen consumption up to and somewhat beyond that in the first hour.

In a second series using fresh muscles the potassium was tipped in to the external fluid in the Warburg manometer flask. In the ensuing 2 hr. very little effect was observed using 5 m-equiv. of potassium but a marked effect was obtained with 10 m-equiv., also with 15 and with 20 m-equiv. In a third series the muscles were soaked, primarily, overnight at 0° in Ringer solution containing 120 m-moles of sodium/l. and no potassium so that they could be expected to contain, on the average, 59 m-equiv. of sodium/kg. They were then re-immersed at room temperature for 1 hr. and for 2 hr., the oxygen uptake being examined over these periods. In the first hour there was a very marked increase of oxygen uptake at and beyond 10 m-moles of potassium externally. In the second hour there was some increase in oxygen consumption but it was considerably less than that observed in the first hour.

The oxygen uptake observations were also associated with sodium and potassium analyses conducted at the end of the experiments on the sartorius preparations, control analyses being performed at the same time.

The effects of potassium on the oxygen consumption in these experiments would seem to be largely explicable by the increased oxygen uptake due to sodium extrusion. Further work in this direction is at present being carried out.

(16) A Study of the Effect on Real Sodium Entrance into Skeletal Muscle Fibres of the Forces Pulling the Sodium Inwards

By Dorothy M. Conway and M. G. Harrington. (*Department of Biochemistry and Unit for Cell Metabolism, University College, Dublin*)

In order to study the real Na^+ entrance into skeletal muscle fibres the active energy requiring efflux of Na^+ must be fully inhibited. The inhibition can be brought about at room temperature by incorporating iodoacetate or Ouabain in the immersing fluid or simply by working at low temperatures. The latter method has two distinct advantages, in delaying degeneration of the muscle and in being without apparent effect on membrane permeability. The extent of uptake of Na^+ depends on the osmotic and electrical forces exerted across the membrane. The former can be considered to depend on the concentration gradient of Na^+ across the membrane, the latter on the membrane potential. Variations in these forces can be brought about by varying the external concentrations of Na^+ and K^+ respectively.

Frog sartorii were immersed in triplicate at approx 4° in Ringer–Conway fluids containing Na^+ at concentrations of 120 and 104 mM with K^+ levels ranging from zero through 5 and 10 mM to 20 mM for periods of up to 24 hr., after which they were weighed and analysed for Na^+ and K^+. It was found that the uptake of Na^+ took the form of a rapid initial phase of up to 3 hr. duration, followed by a slower phase to 24 hr. The membrane potentials corresponding to the electrolyte analyses were determined on muscles immersed under identical

conditions. It has been sought to interpret the first phase of Na^+ entrance in relation to the forces operating and with reference to the equation integrating the Nernst differential equation and the assumption of a constant field. The results were discussed on that basis.

(17) Phycoerythrobilin, a Prosthetic Group of Algal Chromoproteins

By C. Ó hEocha, P. Ó Carra and D. Carroll. (*Department of Chemistry, University College, Galway*)

R-phycoerythrin contains two kinds of bile pigment prosthetic groups (Ó hEocha & Ó Carra, 1961). One, phycoerythrobilin, is obtainable as a hydrochloride after acid hydrolysis of the chromoprotein for 20 min. at room temperature (Ó hEocha, 1958). The spectral and other properties of phycoerythrobilin, which was also obtained from phycoerythrins other than the R-variety, differ from those attributed to the bilidiene, mesobilirhodin (Siedel, 1935; Lemberg & Legge, 1949). Further study indicates that, as originally proposed by Lemberg & Bader (1933) phycoerythrobilin is a bilirhodin, since its properties are best accounted for in terms of the rhodin structure proposed by Siedel (1935).

Phycoerythrobilin is oxidized by ferric chloride to a mesobiliverdin, and is isomerized by acid to violinoid and urobilinoid pigments. Because of its lability the spectral properties of phycoerythrobilin are not known with certainty, but its hydrochloride has $\lambda_{max.}$ at shorter wavelengths (576 mμ in $CHCl_3$; about 557 mμ in 2N-HCl) than mesobiliviolin–HCl and at longer wavelengths than urobilin–HCl. The $\lambda_{max.}$ of its zinc-complex salt is also intermediate between those of the mesobiliviolin and urobilin zinc-complexes. These properties imply that the number of conjugated double bonds in phycoerythrobilin lies between that of the violinoid (8) and urobilinoid (5) systems. The only structure which appears to account for all its properties is that proposed by Siedel (1935) for mesobilirhodin; it contains seven conjugated double bonds. The structure proposed by Lemberg & Legge (1949), which contains five conjugated double bonds, may represent the isomeric urobilin rather than the rhodin.

A mesobilirhodin prepared by Gray & Nicholson (1958; personal communication) by isomerization of (+)-urobilin under alkaline conditions is closely related to phycoerythrobilin. The spectra of the two pigments correspond in acid–chloroform solution ($\lambda_{max.}$ 312 and 576 mμ) and those of their orange-fluorescing zinc-complex salts are similar ($\lambda_{max.}$ about 583 mμ, with an additional smaller absorption peak at 540 mμ in the case of the mesobilirhodin zinc-complex). Both pigments are unstable in concentrated hydrochloric acid, forming urobilinoid and violinoid pigments, but they differ in that phycoerythrobilin forms a relatively greater amount of the latter.

The pronounced discrepancy between the properties of phycoerythrobilin, as described here and as reported by Lemberg & Bader (1933), may be due to the presence in their preparation of urobilinoid isomerization products of phycoerythrobilin. The second prosthetic group of R-phycoerythrin is a urobilin, and may arise by isomerization of phycoerythrobilin *in vivo*. It was not released from the protein under the hydrolytic conditions we employed.

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(18) Vitamin A Acid and Reproduction in Male Rats

By J. N. Thompson, J. McC. Howell and G. A. J. Pitt. (*Departments of Biochemistry and Veterinary Pathology, University of Liverpool*)

Rats grow well and appear normal when fed a diet deficient in vitamin A alcohol, but supplemented with vitamin A acid, the corresponding carboxylic acid. They are however blind (Dowling & Wald, 1960).

We have recently found (Thompson, Howell & Pitt, 1961) that female rats on such a diet become pregnant but resorb the foetuses. The investigations reported here show that in male rats deficient in vitamin A, vitamin A acid will not maintain spermatogenesis.

Weanling male rats were fed one of four diets: (1) basal diet deficient in vitamin A; (2) basal diet + vitamin A alcohol (3·4 μg. of acetate/g. of diet); (3) basal diet + vitamin A acid (10 μg. of methyl ester/g. of diet); (4) basal diet + vitamin A acid (increasing from 10 to 500 μg./g. during the experimental period).

After 5 weeks on these diets, abnormalities were seen in the reproductive system of the completely deficient animals and of those fed vitamin A acid. The changes then rapidly advanced. The earliest sign was a fall in the number of spermatozoa in the epididymis. This was accompanied by a marked increase in the number of sloughed cells present. In the later stages no spermatozoa were present in sections of the epididymis. In the testes there was a reduction in the number of cells lining the tubules and eventually the tubules appeared to be lined by Sertoli cells only.

Animals on the higher doses of vitamin A acid developed multiple bone fractures due to hypervitaminosis A and had to be killed before most of the others, but the early testicular changes were plainly visible.

Those receiving non-toxic doses of vitamin A acid showed normal growth and external appearance; the completely deficient animals reached a growth plateau after 4 weeks on the diet and had severe xerophthalmia when killed 3 weeks later. This work, together with earlier findings on female rats (Thompson, Howell & Pitt, 1961), shows that the role of vitamin A in reproduction in both sexes is different from its so-called 'systemic' mode of action, i.e. concerned with growth and general tissue maintenance.

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(19) The Biological Evaluation of Wheat Proteins

By M. G. HARRINGTON (*Department of Biochemistry, University College, Dublin*)

A comparatively simple means of assessing the biological value of foodstuffs was sought. To this end we have made a comprehensive study of the growth characteristics of our rat colony. Growth of groups of weanling rats was followed for periods of up to 6 weeks on a series of diets and a growth rate for each diet was established. Diet *A*, a standard laboratory diet, available commercially in meal form and containing mixed vegetable protein, carbohydrate fat and fibre in balanced proportions, was found to support a growth rate of 2·25 g./day—'a standard growth rate'. Diet *B*, a modification of diet *A*, was prepared by supplementation with animal protein and fat material, vitamins and mineral salts, to give a 'maximum growth rate' of 2·50 g./day, which was not exceeded by further supplements. In diet *C*, prepared in the laboratory, the entire protein content was supplied by whole wheat, all non-protein constituents being supplied by supplements similar to those used in diet *B*. Growth observed on this diet was referred to as the 'test growth rate'. By studying the effect of alternating these diets at weekly intervals it was found that the animals rapidly adapted to such changes in diet and established the relevant growth rate immediately. This reflexion of variations of diet in rat-growth rates was taken to indicate that a comparison of the 'growth-promoting properties' and hence the biological values at least of related foodstuffs could be made using this system. The relative biological values of a series of wheat samples were assessed by determining the growth rates produced on a diet of type *C* above prepared from each sample; these were then expressed as a percentage of the maximal growth rates. These values were related to the strain of wheat, its treatment during growth and the properties of the grain associated with its milling characteristics.

(20) Nucleotide Degradation in Frozen Cod (*Gadus callarias*) Muscle

By N. R. JONES and J. MURRAY. (*Torry Research Station, Department of Scientific and Industrial Research, Aberdeen*)

It has been known for some time that changes in nucleotide composition may accompany the slow freezing of fish muscle (e.g. Saito & Arai, 1957). We have now examined the course of nucleotide degradation in cod muscle which has been rapidly frozen in liquid nitrogen and stored subsequently at subzero temperatures. The fish were trawl-caught and stored whole, subsequently to freezing, at −78° for 24 hr. aboard ship. Some were then analysed as 'controls' and others stored at −14°, −20° or −30°, protected from evaporation of muscle moisture by an ice 'glaze'.

The fish had struggled considerably in the trawl and the controls contained (per g., means of 6) 0·238 μmole of adenosine triphosphate, 0·369 μmole of adenosine diphosphate, 0·586 μmole of adenosine monophosphate, 3·95 μmoles of inosine monophosphate and 0·159 μmole diphosphopyridine nucleotide, as measured by quantitative elution from Dowex-1 (formate) resin.

Considerable changes were observed at the three storage temperatures. Unlike experiments at 0° (Jones & Murray, 1961) the glycolytic system was active in increasing adenosine triphosphate concentrations—to a maximum of 1·53 μmoles/g. at −14° after 2 weeks, 1·68 μmoles/g. after 15 weeks at −20°, and 1·31 μmoles after 15 weeks at −30°. Thereafter values fell. A stable level at about

0·26 μmole was found after 15 weeks at −14°, corresponding to a residual bound nucleotide fraction reported earlier for experiments at 0° (Jones & Murray, 1961). At this temperature the concentration of diphosphopyridine nucleotide had also fallen to a stable value (0·012 μmole/g.) after 15 weeks, whereas levels of inosine monophosphate increased. Changes at the lower temperatures were slower.

Of the products of nucleotide cleavage, inosine increased in concentration from an initial 0·047 μmole/g. to 3·01 μmoles/g. after 62 weeks at −14° and to 1·08 μmoles/g. at −20°. There was little change at −30°. Hypoxanthine increased from trace values to 0·569 μmole/g. at −14°, but there was relatively little change at the lower temperatures. Guanine appeared in large quantities (0·88 μmole/g. after 62 weeks) at −14° relative to that estimated in cod stored at 0°.

Thus the patterns of nucleotide degradation in frozen muscle differ considerably from that found in cod maintained at 0°. There are also differences between the relative activities of the different degrading systems at different subzero temperatures.

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(21) Nucleic Acid Turnover and Alterations in Ribosomes during Enzyme Induction in Resting Bacteria

By M. H. Proctor.* (*Department of Biochemistry, University of Cambridge*)

In resting bacteria induction of *o*-nitrophenyl-β-D-galactosidase (ONPGase) by melibiose is accompanied by rapid ribonucleic acid turnover, which precedes enzyme appearance in the cells (Proctor, 1960). The ribonucleic acid which is rapidly synthesized has been localized and its presence tentatively related to the ribosome modifications which accompany induction.

Cells grown in mineral salts medium (Proctor & Wilson, 1958) were suspended at 2 mg. dry wt./ml. in 0·1 M-phosphate, pH 7·0, and shaken at 30°. After 30 min. with [^{14}C]guanine, [^{14}C]uracil or [^{14}C]adenine present, either distilled water or 0·01 M-melibiose was added to samples of the suspension. After a further 60 min. the cells were centrifuged and washed twice in pH 7·5 TS2M buffer, which contains 10 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris), 4 mM-succinic acid, and 10 mM-magnesium acetate. The washed cells were resuspended in TS2M and broken in a pressure cell. After centrifugation of unbroken cells and large debris from the cell juice, ribosomes (144 P120; Alexander, 1956) were separated from the soluble fraction (144 S120), and these fractions monitored for trichloroacetic acid-precipitable ^{14}C-labelled compounds. In both induced and non-induced cells equal amounts of ^{14}C were present in the 144 S120 fraction, but only in induced cells were the ribosomes radioactive.

Ribosomes were fractionated by centrifugation of 0·1 ml. of clarified cell juice through a linear gradient of 5 to 20% (w/v) sucrose in 4·5 ml. of TS2M. Non-induced resting cells contained one major ribosome type with sedimentation coefficient (uncorrected) of 85s, and small amounts of 70s and 50s particles; in contrast, induced cells contained larger amounts of the 70s and 50s particles.

Although after 60 min. induction, cells were not yet producing ONPGase, all the [^{14}C]guanine incorporated into the 144 P120 during induction was associated with the 70s ribosomes. In parallel experiments with ^{14}C- or ^{3}H-labelled amino acids the ribosomes were non-radioactive.

Cells were induced for 90 min., and during linear production of ONPGase, ribosomes were prepared in TS2M, washed thrice by centrifugation through TS2M, and fractionated in the sucrose gradient. The ONPGase not in the 144 S120 was associated with the 70s ribosomes. This enzyme activity, increased six-fold by incubation of the ribosomes with protamine sulphate or ribonuclease for 30 min. prior to enzyme assay, could indicate that ONPGase is 'nascent' on the 70s ribosomes (McQuillen, Roberts & Britten, 1959; Cowie, Spiegelman, Roberts & Duerksen, 1961).

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* Overseas Science Research Scholar of the Exhibition of 1851.

(22) Sulphite Reduction by Yeast Enzymes

By T. Wainwright. [*Chemist's Laboratory, Arthur Guinness Son and Co. (Dublin) Ltd., Dublin*]

Some strains of *Saccharomyces cerevisiae* form sulphide from sulphite during growth (Tanner,

1918; Urion & Chevalier, 1946; Zambonelli, 1958) and crude yeast extracts reduce sulphate to sulphite and sulphide (Hilz, Kittler & Knape, 1959). The enzymes involved in the reduction to sulphite have been partially purified (Bandurski, Wilson & Asahi, 1960; Hilz & Kittler, 1958), but the reduction of sulphite has been studied less. Lezius is reported (Gregory & Robbins, 1960) to have obobtained, from yeast, an unstable preparation requiring only reduced triphosphopyridine nucleotide to reduce sulphite to sulphide.

The brewer's yeast used in the present work forms very little sulphide from sulphite during growth or in non-growing suspensions, but extracts readily reduce sulphite to sulphide. The extracts have been purified sufficiently to show that at least two enzymes are required.

One enzyme is comparatively stable. It withstands heating to 55°, acidification to pH 5 and prolonged dialysis with distilled water. It is not affected by treatment with ethylenediaminetetraacetic acid. The enzymic activity is increased over 100-fold by treatment with ammonium sulphate acetone, and calcium phosphate gel.

The other enzyme preparation is much less stable. It can be dialysed against 0·2M-phosphate buffer, pH 7·6, but not 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol or 0·02M-phosphate buffer at the same pH. It is rapidly inactivated at room temperature and at pH values below 6 or above 8. It has been purified slightly by fractionation with ammonium sulphate, protamine sulphate, calcium phosphate gel and diethylaminoethylcellulose.

The combined enzymes reduce sulphite or dithionite to sulphide but neither is active alone. The only substances required by the dialysed enzymes are phosphate buffer, sulphite and reduced triphosphopyridine nucleotide. Neither enzyme reduces colloidal sulphur but each (particularly the less purified) produced hydrogen sulphide from such sulphydryl compounds as cysteine and reduced glutathione.

Unsuccessful attempts were made to detect an intermediate by incubating with either enzyme missing, then adding the other enzyme after inactivating the one originally present.

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(23) *S*-Methylcysteine and *S*-Ethylcysteine in the Sulphur Metabolism of Yeast

By G. A. Maw. [*Chemist's Laboratory, Arthur Guinness Son and Co. (Dublin) Ltd., Dublin*]

Previous studies on the utilization of the sulphur of various compounds by a brewer's yeast (Maw, 1960, 1961*a*) have shown that *S*-methyl-L-cysteine can act as a source of sulphur for growth, although not as effectively as sulphate or L-methionine. In contrast, *S*-ethyl-L-cysteine was completely unable to act as a sulphur source. The behaviour of the above compounds may be compared with that of L-cysteine, which at levels above about 0·78 mM caused depression of growth.

During an examination of the inhibition of growth of the yeast by L-ethionine (Maw, 1961*b*), it was found that *S*-methyl-L-cysteine is one of a small number of compounds which can annul this growth inhibition. *S*-Methylcysteine is approximately one-fifth as effective as L-methionine in this respect. *S*-Ethyl-L-cysteine, however, is ineffective in annulling the growth inhibition and, moreover, under certain conditions is itself growth-inhibitory. This growth inhibition, like that caused by ethionine, can be abolished by L-methionine and by *S*-methyl-L-cysteine.

Studies of the uptake of ^{35}S-labelled *S*-methyl-L-cysteine and *S*-ethyl-L-cysteine from a glucose medium by the yeast have been carried out to determine to what extent the above findings might be explained as competitive cell penetration effects. *S*-Methyl[^{35}S]cysteine (0·156 mM) was rapidly taken up from the medium for a period of 60–90 min., but after this time a large fraction of the ^{35}S was released back into the medium. This is in contrast to the slower but continuous uptake of $Na_2{}^{35}SO_4$ by the yeast. *S*-Ethyl[^{35}S]cysteine behaved in a similar manner to *S*-methyl[^{35}S]-cysteine, except that the rate of uptake was slower. The form in which the sulphur of the two cysteines passes back into the medium is being investigated. The uptake of 0·156 mM-*S*-methyl[^{35}S]cysteine over a 1 hr. period was unaffected by the presence of concentrations up to tenfold molar of sulphate, L-cysteine, L-methionine or reduced glutathione, but 1·56 mM-*S*-ethylcysteine reduced the uptake by 55%. The uptake of 0·156 mM-*S*-ethyl[^{35}S]-cysteine, however, was only reduced by 27% by 1·56 mM-*S*-methylcysteine and was unaffected by L-methionine at this concentration.

It is suggested that as well as being able to depress to some extent the uptake of *S*-methylcysteine, *S*-ethylcysteine may act as a growth inhibitor by competing for various intracellular reactions of methionine, either directly or after conversion into ethionine. *S*-Methylcysteine may abolish the growth-inhibitory effects of ethionine

and *S*-ethylcysteine by acting as a precursor of methionine in the yeast, rather than by blocking the uptake of the ethyl compounds.

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(24) *In vitro* Sulphation of Hydroxylated 1:4-Pyrones

By Brian Spencer and D. K. Watkins. (*Departments of Biochemistry, Trinity College, Dublin and University College, Cardiff*)

Chromatograms of incubation mixtures of sheep trachea epithelial cell suspensions in a modified Krebs medium containing $Na_2{}^{35}SO_4$ showed a strong radioactive spot which was distinct from $^{35}SO_4{}^{2-}$. Incubation of the individual components of the Krebs medium with the epithelial cell suspension, adenosine triphosphate and $Na_2{}^{35}SO_4$ showed that the unknown spot was formed only from pyruvic acid. The $Na_2{}^{35}SO_4$ and adenosine triphosphate could be replaced by adenosine 3-phosphate 5′[^{35}S]-sulphatophosphate (PAP^{35}S) which was normally formed in the incubation mixture. When triple-distilled pyruvic acid was used, the radioactive spot was virtually absent, but if the pyruvic acid was allowed to stand for 2 days at room temperature before incubating in the test system appreciable quantities of the unknown spot were formed. The evidence suggested that the unknown spot was a sulphate ester formed by enzymic transfer of sulphate from PAP^{35}S to an impurity which arose when pyruvic acid was allowed to stand.

Optimum conditions for the formation of the impurity appeared to be the refluxing of pyruvic acid at 165° for 2 hr. Testing for the presence of the impurity by its ability to form the unknown sulphate ester in the *in vitro* system, the treated pyruvic acid was fractionated to give colourless crystals of the impurity. The crystals had the composition $C_7H_8O_3$ and were shown to be identical with an authentic sample of 3-hydroxy-2:6-dimethyl-1:4-pyrone. The sulphate ester of this compound was synthesized and found to be chromatographically indistinguishable from the unknown radioactive spot.

Particle-free preparations of tissues from other animals, including rat liver and kidney, were also able to transfer sulphate from PAP^{35}S to 3-hydroxy-2:6-dimethyl-1:4-pyrone as well as to a number of related hydroxylated pyrones. Amongst the compounds sulphated by the animal preparations was kojic acid, but although many fungal preparations could synthesize PAP^{35}S and transfer the sulphate from the PAP^{35}S to choline no transfer to kojic acid or to other hydroxylated pyrones could be detected.

(25) Some Properties of Human-Tissue Lactic Dehydrogenases

By J. H. Wilkinson, K. B. Cooke, B. A. Elliott and D. T. Plummer. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

A comparative study of the rates of enzymic reduction of α-oxo acids by rabbit skeletal muscle preparations and by crystalline ox-heart lactic dehydrogenase showed that in the presence of reduced diphosphopyridine nucleotide greatest activity was exerted against pyruvic and α-oxobutyric acids (Meister, 1949, 1950). Subsequently human serum was found to exert activity against α-oxobutyrate which in several respects differed from that against pyruvate (Rosalki & Wilkinson, 1960).

As zone electrophoresis has shown serum lactic dehydrogenase to be present in several different fractions, and as Plagemann, Gregory & Wróblewski (1960) have found rabbit and human tissues to contain lactic-dehydrogenase activities with different electrophoretic mobilities, we investigated the activities of fractions from different tissues against both substrates. The relative activities $\left(\frac{\text{activity against pyruvate}}{\text{activity against α-oxobutyrate}}\right)$, determined under optimal conditions of substrate concentration, varied from 0·85 in fractions from rabbit- and human-heart muscle having an electrophoretic mobility similar to that of an α_1-globulin, to 3·15 in the γ-globulin fraction obtained from an extract of human liver, and to 5·35 in an extract of rabbit skeletal muscle.

Over-all activities of crude Sørensen phosphate buffer extracts (pH 7·4) of various human tissues showed variations in relative activities similar to those found in the electrophoretic fractions. Over a wide range of concentration of extracts, linear responses were obtained against both substrates, thus indicating the absence of significant quantities of activators or inhibitors.

Determination of the Michaelis constants showed that the affinity for pyruvate was much greater than that for α-oxobutyrate in all the tissues studied, but human-heart extracts showed a much greater affinity for α-oxobutyrate than human-liver extracts. This offers an explanation for the difference between the serum activities found in heart and liver disease (Elliott & Wilkinson, 1961).

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(26) The Relation Between the Composition of the Fatty Acids in the Diet and in Faeces

By Sheila M. Gompertz and H. G. Sammons. (*Little Bromwich Hospital, Birmingham* 9)

Four normal subjects were given a constant low-fat diet for 1 week together with 65 g. of a fairly unsaturated fat ('Spry') daily. The total fatty acids were extracted from 24 hr. stool samples and prepared for analysis by gas-phase chromatography by the method of Sammons & Wiggs (1960). Although some individual variation in the composition of the faecal fats was found, the 'apparent absorption' of the major fatty acids, [(wt. in diet − wt. in faeces) × 100 %/wt. in diet] was qualitatively similar for each subject; that is, oleic acid > palmitic acid > stearic acid. This confirmed the observations of Sammons, Wiggs & Frazer (1961) that it is the proportion of stearic acid in the faeces which is mainly responsible for the differences between dietary and faecal fat.

Holt, Tidwell, Kirk, Cross & Neale (1935) and Mattil (1946) have suggested that stearic acid is more poorly absorbed than other fatty acids. The work of Blomstrand (1955) suggests that conversion of oleic into stearic acid is responsible for the low 'apparent absorption' of stearic acid. A 'labelled' dietary fat, prepared by randomly esterifying 'Spry' with erucic acid (36 %), was fed to the same four subjects by replacing the unlabelled fat in the previous diet. In this case, the saturated acid, behenic acid, was not found in the faeces; therefore no hydrogenation of erucic acid occurred although the 'apparent absorption' of stearic acid was still low. These results indicated that the high content of stearic acid in faeces may be due to its selective rejection during absorption, not to its formation from oleic acid. However, Ueno (1925) has shown that oleic acid is more readily reduced by catalytic hydrogenation than erucic acid, so that the possibility cannot be excluded that oleic acid can be hydrogenated in the intestine, while erucic acid remains unchanged. In one subject with steatorrhoea, partial hydrogenation of erucic acid was demonstrated.

A poor absorption of erucic acid was found (only 92–96 %) although the faecal fat was not raised above normal levels; therefore the selective rejection of a specific fatty acid in a triglyceride is possible. Large amounts of this long-chain fatty acid present in some emulsified fat droplets may prevent their breakdown into particles small enough to be absorbed.

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(27) Factors Affecting Faecal Composition—a Comparison of Ileal Discharge and Faeces

By H. G. Sammons. (*Little Bromwich Hospital, Birmingham* 9)

Two comparative studies have been made of the composition of the ileal discharge of a subject after colectomy with the faeces of a normal person. In each investigation, both subjects were on the same diet throughout the experiment. Excreta were collected over consecutive periods of 24 hr. for a period of 1 week. Detailed analyses of the lipids were made and estimations of water, nitrogen and electrolytes.

There were no gross differences in total fatty acid composition between the two types of excreta but there were some small additional fractions of other fatty acids (C_{15}, C_{15} branched) in every specimen of faeces; these were present only in traces in the ileal discharge. They may be bacterial in origin.

Cholesterol excretion in the ileal discharge was much higher than in the faeces. The unsaponifiable component was in crystalline form showing m.p. above 100°; similar material from the faeces was liquid at body temperature. These results confirm the observations of other workers (Gardner, Gainsborough & Murray, 1935; Hellman & Rosenfeld, 1959). Stearic acid percentage was always higher than in the diet. If this is due to hydrogenation of unsaturated acids, then it must take place more quickly than the conversion of cholesterol. Incubation of fresh ileostomy material produced very little change in either lipid component.

The water excretion was about 500 ml./day in the ileal discharge and about 120 ml./day in the faeces. Nitrogen was 2·7 and 1·8 g. respectively.

The average sodium loss in the ileal discharge was about 55 m-equiv./day compared with 6 m-equiv. in the faeces; the potassium was 5 m-equiv. and 13 m-equiv. and the chloride 24 m-equiv. and 1·5 m-equiv. respectively.

These results suggest that there is an active secretion of potassium by the large intestine and a normal mechanism for the absorption of sodium and chloride. Of interest in this respect is the report by Roy & Ellis (1959) of potassium-secreting colonic tumours. Crawford & Brooke (1957) point out that potassium loss in the faeces by patients with ulcerative colitis is corrected by ileostomy. Hypokalaemia is a common feature of chronic diarrhoea and potassium is always higher than sodium in normal faeces.

It would appear that the large bowel affects the cholesterol composition of the faeces but not to any great extent the fatty acid component. It has a marked influence on the water and electrolyte excretion.

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(28) Measurement of Human Blood Pyruvate in Health and Disease

By T. D. R. Hockaday (introduced by L. I. Woolf). (*Department of The Regius Professor of Medicine, The Radcliffe Infirmary, Oxford*)

Separation of the 2:4-dinitrophenylhydrazones of α-oxo acids by paper chromatography in acid systems has been described (Bush & Hockaday, 1960). One such system was light petroleum (b.p. 100–120°)–toluene–acetic acid–water (10:10:17:3, by vol.) and this has been used in the estimation of the pyruvate content of human blood.

Blood was drawn with the precautions previously recommended (Friedemann & Haugen, 1943) and immediately added to 15–20 ml. of 5% (w/v) metaphosphoric acid in a weighed container. The precipitated proteins were separated by centrifugation. 0·1%-2:4-Dinitrophenylhydrazine in 2N-HCl (2 ml.) was added to the supernatant and the pyruvate derivative extracted with ether (Abdel-Tawab, Broda & Kellner, 1959) whence it was further purified by extraction with 3 ml. of 10% (w/v) sodium carbonate. After re-acidification of this aqueous phase, a final ethereal extract was made, evaporated to dryness, and the residue taken up in 0·5 ml. of ethanol; a portion of this ethanolic solution was applied to paper and the pyruvate compound finally purified by developing the chromatogram for 2 hr. with the mobile non-polar phase. The pyruvate derivative occurred as a single spot with R_F 0·40. Its amount was estimated in one of two ways: either by elution with 10 ml. of 0·1M-phosphate buffer, pH 7·4 (Abdel-Tawab *et al.* 1959) from the relevant section of the chromatogram and measurement of the concentration of the resulting solution with a Unicam spectrophotometer at 390 mμ; or by scanning the chromatogram in a spectrophotometer with automatic graphical recording of the transmitted light.

Analyses on separate samples of blood drawn at one venepuncture varied by ±4%. The recovery of pyruvate, added as its sodium salt [prepared according to Robertson (1942)] to the mixture of blood and metaphosphoric acid, was 92% ± S.D. 3%. The average value of pyruvate in venous blood from normal humans (aged 16 to 56 years), after an overnight fast, was 0·32 ± S.D. 0·08 mg./100 ml. (range 0·19–0·54). Pyruvate metabolism tests (Joiner, McArdle & Thompson, 1950) were carried out as described by Williams, Mason, Power & Wilde (1943). Average values in normals for pyruvate were 0·47 ± S.D. 0·11 mg./100 ml. (range 0·29–0·65) at 60 min. and 0·56 ± S.D. 0·13 mg./100 ml. (range 0·40–0·78) at 90 min. These values are lower than those obtained by classical methods (Friedemann & Haugen, 1943), but are similar to some results obtained by chromatographic separation in alkaline systems (e.g. Cavallini, Frontali & Toschi, 1950; Earl, el Hawary, Thompson & Webster, 1953; Hennes, Wajchenberg, Fajans & Conn, 1957).

Values from a small number of patients were discussed. Their illnesses included peripheral neuropathy, vitamin B_1 deficiency, vitamin B_{12} deficiency, thyrotoxicosis, pancreatic islet-cell adenoma, diabetes mellitus, and McArdle's syndrome.

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(29) The Coagulative Factors of Normal Human and Human Cancer Tissues

By W. A. Boggust, R. A. Q. O'Meara and R. D. Thornes. (*Departments of Biochemistry and Pathology, Trinity College, Dublin*)

O'Meara (1958, 1960) and O'Meara & Jackson (1958) have shown that the coagulative property which differentiates cancer tissues from normal tissues is due principally to the presence of specific water-soluble thermolabile coagulative factors present only in cancers, and to a lesser extent in normal colon and foetal membranes.

The technique employed for the identification and estimation of the thermolabile coagulative content of tissue extracts and fractions was adapted from that of O'Meara (1958) and O'Meara & Thornes (1961). The calcium content was reduced, thereby increasing the sensitivity of the coagulation system. Coagulation times of both plasma buffer controls and test solutions, before and after heating for 30 min. at 60°, were completely reproducible. When plotted against increasing dilutions of tissue extracts, calibration curves were obtained from which the approximate thermolabile activities of fractions were estimated.

Several mono- and di-valent metals were shown to accelerate or inhibit coagulation at precise concentrations in the reaction mixtures. Manganese promoted clotting of plasma buffer substrate. Magnesium activated a coagulation inhibitor which was adsorbed from normal plasma by alumina.

Ammonium sulphate flocculation at pH 7 of aqueous extracts of foetal membrane demonstrated two thermolabile coagulation inhibitors and two distinct thermolabile coagulative factors. These findings are in agreement with the results of starch-block electrophoresis obtained by O'Meara & Thornes (1961).

Column chromatography of tissue extracts on diethylaminoethylcellulose with stepwise elution at pH 9·0 by ammonium acetate of increasing molarity gave satisfactory separation of main protein peaks with little nucleic acid material. Thermolabile coagulative activity was closely associated with certain peaks but was not proportional to protein concentration. Extracts of foetal membrane and of cancers demonstrated two major peaks of thermolabile activity, and a third labile peak which was less marked in membrane. Comparable normal tissue extracts of similar protein content demonstrated only traces of thermolabile activity almost entirely non-coincident with major peaks characteristic of cancer or membrane.

The principal thermolabile peak is eluted by 0·4M-ammonium acetate and appears to be characteristic of carcinomas. It may be identical with a prealbumin thermolabile coagulative factor, prepared by electrophoresis referred to above. This factor is present in all cancer tissues, and also in foetal membrane and normal colon, but is not present in other normal tissues.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **407th Meeting** *of the Society was held in the Department of Physiology and Biochemistry, University of St Andrews, on Thursday and Friday,* 22 *and* 23 *June* 1961, *when the following papers were presented:*

COLLOQUIUM ON 'THE QUANTITATIVE ESTIMATION OF AMINO ACIDS'

The Method of Moore and Stein

By J. E. Eastoe.* (*Department of Dental Science, Royal College of Surgeons, London, W.C.* 2)

Ion-exchange resins have made possible methods for amino acid analysis which are both more convenient and also capable of readily resolving more complex mixtures than earlier quantitative chromatographic methods using silica gel (Tristram, 1946) and starch (Moore & Stein, 1949). Sulphonated polystyrene resins have been mainly used. Originally the amino acids were eluted in cationic form with increasing concentrations of hydrochloric acid (Stein & Moore, 1950) but improved results were subsequently obtained by using successive buffers of increasing pH, within the range 3 to 7, where the amino acids exist as an equilibrium mixture of cations and zwitterions (Moore & Stein, 1951). Separation and quantitative recovery of amino acids from 5 to 10 mg. of protein were effected with two columns of 8 % cross-linked resin. Analysis of 3 mg. of protein on a single column was made possible by the introduction of concentration and pH gradients in the buffer system (Moore & Stein, 1954).

A lower degree of cross-linking renders the resin matrix sufficiently open for chromatography of small peptides (Dowmont & Fruton, 1952), but volume changes on regeneration necessitate repacking the column for each analysis. Distribution of resin particle size affects chromatographic efficiency. Hamilton (1958) introduced a hydraulic back-washing method of fractionation and showed that irregular particles of accidental diameter 30 μ were equal in efficiency to 25 μ-diameter spheres. Fine resins, eluted under pressure, allow faster flow rates while maintaining sharp peaks, thus enabling analyses to be completed more quickly. This facilitated the development of automatic methods of amino acid analysis (Moore, Spackman & Stein, 1958; K. A. Piez, personal communication). Reduction of column diameter and fraction volumes permit analyses on 0·3–0·5 mg. of protein (Eastoe, 1961).

Accuracy is affected by many factors, including purity of sample and standards, hydrolysis conditions, assessment of the base line, peak overlap, undetected substances, reproducibility of colorimetry, fading and stability of the ninhydrin reagent. Collaborative tests (Bender, Palgrave & Doell, 1959) showed that experienced workers obtained results with an average error of ± 3 % but in some laboratories deviations were greater. The accuracy for different amino acids varied considerably. The automatic method should remove several sources of observational error.

Difficulties in establishing the method could be minimized if materials conforming to suitable specifications were available. This applies particularly to resin, amino acids, and reagents such as ninhydrin, methoxyethanol, thiodiglycol, detergents and buffer acids.

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Some Aspects of Automation

By A. L. J. Buckle. (*Department of Experimental Medicine, Guy's Hospital Medical School, London, S.E.* 1)

The chromatographic separation of amino acids on cation-exchange resin columns was successfully automated by Moore, Spackman & Stein (1958) after Hamilton (1958) had developed a method of separating ion-exchange resins into fractions of narrow particle-size distribution. Their system embodied square wave changes of pH during development and elution and the separation of acid, neutral and basic fractions on different columns at column pressures of 30–50 lb./sq.in. The necessity for the use of programmed solenoid

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valves to effect the changes in buffer pH can be avoided by employing a pH gradient device after Peterson & Sober (1959) and in many cases this permits the separation of acid, neutral and basic amino acids on one column with very careful selection of resin particle size and degree of cross-linking. Certain resin fractions permit the operation of 130 cm. columns at pressures as low as 10 lb./sq.in. and a peristaltic pump can be employed for column development, as in the Autoanalyzer system which also employs nitrogen segmentation of the liquid streams during colour development.

The minimum time of 24 hr. at present required for a complete chromatogram may be shortened by utilizing the results of the work of Hamilton (1960) on multi-section columns using high pressure and flow rates, and it may also be possible to store effluent from several columns in large time-delay coils to be analysed later at high speed. Recent work on the gas-liquid chromatography of amino acid derivatives may also render feasible a complete separation in a few hours.

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Rapid Methods for the Determination of Glucosamine, Galactosamine and Hydroxyproline

By S. M. Partridge and D. F. Elsden. (*Low Temperature Research Station, Downing Street, Cambridge*)

Hydroxyproline. The method is suitable where the hydroxyproline content of the sample is low or where direct use of the Newman & Logan (1950) reagents is undesirable because of interference by tryptophan or tryosine. The column, 0·9 cm. diameter, was packed with Amberlite G.C. 120 resin to a height of 4 cm. The resin used was a fraction with particle diameter $40 \pm 7\,\mu$, obtained by hydraulic classification as described by Moore, Spackman & Stein (1958). The column was equilibrated with 0·2M-sodium citrate buffer of pH 2·5; addition of phenol, thymol or detergent was avoided because of interference with the colorimetric readings. The sample of protein containing about 0·2 mg. of hydroxyproline was sealed in a tube with 1 ml. of 6N-HCl and hydrolysed at 100° for 40 hr. A portion (0·2 ml.) of the hydrolysate was applied directly to the column without prior removal of acid. The column was eluted at 20° with the same buffer (flow rate, 20 ml./hr.) and 1 ml. fractions collected for 90 min. The hydroxyproline peak appeared in fractions 26 and 27 and was estimated using the reagents of Newman & Logan (1950). Replicate recoveries of hydroxyproline from gelatin were within 1% of the average values given by the standard Moore & Stein procedure.

Hexosamines. Economy of time and material was again obtained by applying the acid hydrolysate directly to the column. The column (30 cm. × 0·9 cm.) was packed with the same resin fraction and was equilibrated with 0·35M-sodium citrate buffer of pH 5·26. The mucoprotein sample containing about 0·5 mg. of total hexosamine was sealed with 1 ml. of 4N-HCl and hydrolysed for 16 hr. at 100°. A portion (0·2 ml.) of the hydrolysate was applied to the column which was then eluted with the same buffer at 50°; flow rate, 20 ml./hr.; volume of fractions, 2 ml. Glucosamine appeared in fractions 25–27 and galactosamine in fractions 27–30; the peaks were sufficiently well separated to apply the usual correction for overlap. The ninhydrin reagent of Yemm & Cocking (1955) was used to estimate hexosamine; if hydroxylysine is present it overlaps the galactosamine peak and a longer column should then be used. The estimates should be corrected for loss of glucosamine and galactosamine during hydrolysis, but these substances are recovered quantitatively if added to the protein hydrolysate.

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End-Group and Sequence Studies on Proteins

By J. I. Harris.* (*Department of Biochemistry, University of Cambridge*)

In spite of the considerable advances in experimental methods which have followed in the wake of Sanger's pioneer studies on insulin (Sanger, 1956), the study of amino acid sequences in proteins has not yet reached the stage where it is possible to determine the complete amino acid sequence of any given protein by standard routine methods.

In general terms there are two main avenues of approach to the problem of sequence analysis. One

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way is to undertake a series of qualitative degradative experiments involving hydrolysis with proteolytic enzymes of differing specificities such as trypsin, chymotrypsin, pepsin, subtilisin and papain, followed by the fractionation and chemical characterization of the resulting polypeptide fragments by 'paper' methods.

Many factors such as the availability, chemical homogeneity, molecular size, and overall amino acid composition of the starting material play a decisive role in determining how a particular polypeptide or protein is to be tackled, and qualitative paper methods are particularly suitable in the case of precious biologically active substances which occur in small amounts and which are difficult to isolate in analytically pure form; for example, pituitary polypeptide hormones, such as corticotropin and melanocyte-stimulating hormone (Harris, 1960), and hypertensin (Elliott & Peart, 1957). On the other hand, it would be extremely difficulty to attempt to determine the complete amino acid sequence of larger proteins [for example, ribonuclease (Hirs, Moore & Stein, 1960)], or tobacco mosaic virus protein (Anderer, Uhlig, Weber & Schramm, 1960; Tsugita *et al.* 1960) in this way. At this level of complexity paper methods by themselves are not suitable for the purification of peptide fragments on a sufficiently large scale, and it would be extremely hazardous to rely exclusively on qualitative methods for subsequent sequence studies.

The development of ion-exchange chromatography for fractionating peptide fragments produced by enzymic hydrolysis of proteins, and of automatic equipment for quantitative amino acid analysis, greatly facilitated the elucidation of the amino acid sequence of ribonuclease, a protein consisting of 124 amino acids (Hirs, Moore & Stein, 1960). In this case it was found possible to deduce a partial structure, in which the relative order of the peptide fragments was known, simply by determining the amino acid compositions and end groups of peptides isolated from tryptic and chymotryptic digests of the protein. Unfortunately subsequent experience has shown that even this elegant quantitative approach cannot be regarded as a general 'recipe' for the determination of amino acid sequences. Thus, in the case of other proteins, for example, tobacco mosaic (Anderer *et al.* 1960; Tsugita *et al.* 1960), and turnip yellow mosaic (Harris & Hindley, 1961), virus proteins, and cytochrome *c* (Margoliash, 1961), it has been found necessary to use paper methods, for refractionating peptide 'peaks' from ion-exchange columns, and, in conjunction with quantitative amino acid analysis, for establishing overlapping sequences along the protein chain.

Each and every protein presents a different problem, and it is clearly necessary to be flexible in one's approach and to make use of as many and diverse methods as possible to achieve success.

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The use of Isotopically Labelled Reagents in the Determination of Sub-Microgram Quantities of Amino Acids

By J. K. WHITEHEAD. (*Barnato-Joel Laboratories, Middlesex Hospital Medical School, London, W.* 1)

The method of isotope derivative analysis was first used in the estimation of amino acids by Keston, Udenfriend & Cannon (1946) using ^{35}S- and ^{131}I-labelled *p*-iodophenyl sulphonyl chloride as the labelled reagents. Whitehead (1958), using acetic anhydride labelled with ^{3}H and ^{14}C, extended this technique to the assay of amino acids at levels as low as 10^{-5} micromoles for a particular component of a mixture. Complete amino acid analyses could be made of hydrolysates from only 1 μg. of original protein. This acetylation method had a number of disadvantages and recently Beale & Whitehead (1961) have further extended the technique to the use of 1-fluoro-2:4-dinitrobenzene labelled with ^{3}H and ^{14}C.

The method involves the treatment of the mixture of amino acids with the tritiated reagent (specific activity 25 μC/μequiv.) in alkaline solution followed by the addition of known activities (6000 disintegrations/min.) of ^{14}C-labelled 2:4-dinitrophenyl (DNP) derivatives. After solvent extraction, the mixture is separated into its constituent dinitrophenyl derivatives by two-way paper chromatography and the ^{3}H and ^{14}C contents of each spot determined by combustion and gas counting. The ^{3}H count gives a measure of the amount of DNP-amino acid isolated from the original mixture and the ^{14}C count gives a measure of losses incurred during the analysis.

The results obtained from the analysis of 1 μg. of hydrolysed insulin are in good agreement with values obtained by derivative analysis using

labelled acetic anhydride on a similar amount of protein, and also with values obtained by methods involving milligram quantities. The results obtained by the labelled DNP method are in most cases within 2–3% of the values calculated from the formula of insulin (Ryle, Sanger, Smith & Kitai, 1955).

The DNP isotopic method has also been extended to the determination of *N*-terminal and *C*-terminal groups of proteins using amounts of original material of the order of 1 μg.

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Quantitative Analysis by Paper Chromatography or Electrophoresis

By B. S. Hartley. (*Department of Biochemistry, University of Cambridge*)

The merits of simplicity, sensitivity, flexibility and economy would appear to favour paper chromatography or electrophoresis in quantitative analysis of amino acid mixtures, but despite a considerable literature there is today no established technique. To some extent, the multiplicity of methods reflects the different requirements of the analysts; thus clinical biochemists will value simplicity, enzymologists will seek sensitivity, students of protein structure will look for flexibility and the Treasury will appreciate economy. Where accuracy and reproducibility are prime criteria, ion-exchange methods appear preferable, but paper can offer many other advantages.

Simple visual comparison of the intensity of ninhydrin spots against a series of standards is useful in estimating amino acid ratios in hydrolysates of small peptides. One-dimensional separations by chromatography (e.g. Hanes, Harris, Moscarello & Tigane, 1961) or high-voltage electrophoresis (e.g. Michl, 1959) lend themselves to this approach. The accuracy can be increased by photometric scanning of the ninhydrin spots (e.g. Block, 1950) or of a photographic copy (Keil, 1954).

Measurement of the ninhydrin colour in solution is capable of greater accuracy, but introduces complexities due to the need for improved separation of the amino acids, elimination of interfering impurities in the paper and control of the temperamental ninhydrin colour reaction. Hanes (1961) and Wade, Matheson & Hanes (1961) have investigated these variables and propose two complementary one-dimensional chromatographic systems suitable for the analysis of almost all the common amino acids (Hanes *et al.* 1961). The spots are detected by a dilute ninhydrin dip, ammonia is removed by an alkali treatment and the full colour is developed in solution with an improved ninhydrin-hydrindantin reagent (Hanes *et al.* 1961). A modification of these techniques has assisted the author in protein sequence studies. The main difference is the use of a two-dimensional separation with high resolution which preserves much of the reproducibility of the one-dimensional system by running spots in parallel by electrophoresis and stitching the consequent panels side by side in chromatography (Richmond & Hartley, 1959). A ninhydrin–cyanide reagent (Cocking & Yemm, 1954) has shown advantages in storage, handling and sensitivity to ammonia.

Even these improved techniques cannot be considered completely satisfactory; the basic flaw is the complexity and relative insensitivity of the ninhydrin reaction. Methods involving the reaction of amino acids with copper do not demonstrate decisive advantages, whilst the fluorodinitrobenzene technique of Levy (1954)—though accurate in careful hands—involves considerable correction factors. Moreover, the possibility of sequence work on proteins available only in milligram quantity demands methods of analysis at the micromillimole level. The isotope dilution techniques of Kesten, Udenfriend & Levy (1950) and Whitehead (1958) offer this to the diligent. An alternative approach involving fluorescent conjugates of amino acids with 1-dimethylaminonaphthalene 5-sulphonyl chloride was reported.

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COMMUNICATIONS

(1) Automatic Analysis of Amino Acids by Polarographic Estimation of their Copper Complexes

By M. C. Corfield and A. Robson (introduced by F. O. Howitt). (*Wool Industries Research Association, Leeds* 6)

Polarographic estimation of the copper complexes of amino acids has been used for quantitative analysis of mixtures separated by chromatography on paper (Martin & Mittelmann, 1948), starch (Kemula & Witwicki, 1955) and ion-exchange resin (Blaedel & Todd, 1961). We have recently constructed an apparatus for automatic amino acid analysis using this method of estimation.

The amino acids are separated on cation-exchange resin columns by the method of Spackman, Stein & Moore (1958) with the following modifications. Chloroacetate buffer, pH 3·18, and acetate buffers, pH 4·25 and pH 5·28, are used on 150-cm. and 15-cm. columns of Zeo-Karb 225 (X8) resin, as 20–30 μ beads, instead of the usual citrate buffers, because citrate ion complexes with copper. To facilitate the subsequent adjustment of these buffers to pH 9·2, they have been formulated to contain the same concentrations of free acid. Detergent interferes in the analysis and is omitted from all buffers.

A high-pressure displacement pump delivers buffer at a constant rate to a cation-exchange resin column loaded with an amino acid mixture. Part of the effluent from the column is removed continually and mixed with an equal volume of a neutralizing solution by a two-tube peristaltic pump. The mixed solution, now at pH 9·2, is passed through a thermostat at 30° and down a column (8 cm. × 0·9 cm.) of a mixture (1:1, w/w) of $Cu_3(PO_4)_2$ and Cellulosapulver (ethanolysed cellulose powder, Grycsbo Pappersbruck, Grycsbo, Sweden), which converts amino acids quantitatively into their copper complexes. A stream of nitrogen sweeps the solution into a specially constructed polarographic cell that responds to rapid changes of concentration. This cell is maintained at 30° and has separate outlets for nitrogen, mercury and effluent. A potential difference of 0·7 v is applied between the dropping mercury electrode and the mercury anode. The diffusion current is amplified by a d.c. galvanometer feed-back amplifier and recorded.

Amounts of amino acids are determined from the areas of the corresponding peaks on the record, with reference to data obtained previously with known amounts. Citrulline, which does not normally occur in protein hydrolysates, is added to the amino acid mixture to provide a check on the reproducibility.

A protein hydrolysate containing 1–2 μmoles of amino acids, can be analysed in 48 hr. with a reproducibility of $100 \pm 3\%$. Installation of an automatic solvent-change mechanism should reduce the time for complete analysis to 24 hr.

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(2) A Method for the Estimation of Cystine plus Cysteine in Proteins

By J. C. Fletcher and A. Robson (introduced by F. O. Howitt). (*Wool Industries Research Association, Leeds* 6)

The sulphur chemistry of proteins has recently been critically reviewed by Cecil & McPhee (1959), who emphasize the difficulties involved in the assay of cystine and cysteine in proteins. Methods that give reliable results with intact soluble proteins are less convincing when applied to insoluble proteins, such as wool, and the results obtained by methods based on a preliminary acid hydrolysis may require correction for the decomposition of cystine and cysteine that occurs during hydrolysis.

Acid hydrolysates of ^{35}S-labelled wool have been found by column chromatography to contain, in addition to cystine and cysteine, several unidentified sulphur-containing substances (Lewis, Robson & Tiler, 1960). The same group of ^{35}S-labelled substances is obtained when unlabelled wool is hydrolysed in the presence of [^{35}S]cystine, but not when [^{35}S]cystine is boiled in acid alone. Further, the specific activities of all the other ^{35}S-labelled compounds present are the same as that of the chromatographically isolated cystine. All these compounds must therefore be derived ultimately from cystine, either peptide-bound or free, by decomposition under the influence of, or by reaction with, other substances present in the hydrolysate. The reactions involved may include disulphide interchange, and any such interchange must have reached equilibrium under the conditions of the hydrolysis. These findings suggest that the follow-

ing method be examined for the determination of cystine plus cysteine in proteins.

A measured activity of [^{35}S]cystine is added to a known weight of protein, and the mixture is hydrolysed in 5·7N-hydrochloric acid. A sample of the hydrolysate is chromatographed on a 40 × 0·9 cm. Zeo-Karb 225 (X4) column with 2·5N-hydrochloric acid as eluent. The [^{35}S]cystine is isolated and the specific activity of the sulphur is determined. From this and the [^{35}S]cystine activity originally added, the contribution of cystine plus cysteine to the sulphur content of the protein can be determined.

Preliminary results with insulin are promising, and further work with wool and lysozyme is in progress, the results of which will be reported later.

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(3) On the Determination of Tryptophan in Proteins

By Pauline M. Harrison and T. Hofmann. (*Department of Biochemistry, University, Sheffield* 10)

The method of Spies & Chambers (1948, 1949) is probably the most reliable method available at present for the determination of tryptophan in proteins. It appears to give excellent results with a number of proteins. However, it has been observed [see, for example, Hamilton (1960)] that some proteins, instead of giving a blue colour (absorption maximum 590–600 mμ) like that given by free tryptophan, give a purple-pink colour (absorption maximum 545–560 mμ). We have found that this is the case for human-serum albumin, apoferritin, diaphorase and liver alcohol dehydrogenase. With these proteins no reliable estimate of the tryptophan content can be obtained because of low yields, slow formation and instability of the colour. Stable blue chromophores with essentially the same absorption spectrum as that given by free tryptophan can, however, be obtained, if the proteins are first denatured and partially digested with a mixture of chymotrypsin and trypsin.

Protein samples (0·1–0·25 ml.) containing 5–30 μg. of tryptophan are heated in open test tubes in a boiling-water bath for 15 min. After cooling 0·2M-tris buffer (pH 9·2, 0·1 ml.) is added, followed by 0·02 ml. of chymotrypsin (1 mg./ml.) and 0·02 ml. of trypsin (1 mg./ml.). The mixture is incubated at 37° for periods varying from 30 min. to 17 hr., the time necessary for digestion depending on the individual protein. The volume is made up to 0·25 ml. The *p*-dimethylaminobenzaldehyde-H_2SO_4 reagents of Spies & Chambers (1949) are then added and procedure K of these authors method is followed, except that the reagent volumes were reduced to one-quarter.

Using this method we obtained the following results, given as number of tryptophan residues per mole of protein: human-serum albumin 0·98, apoferritin 20·1, diaphorase 1·95 and liver alcohol dehydrogenase 3·2.

One of us (P.M.H.) acknowledges the receipt of a personal research grant from the Medical Research Council.

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(4) The Role of Soluble Ribonucleic Acid in the Synthesis of Serum Albumin by the Isolated Microsome Fraction from Rat Liver

By Alexandra von der Decken* and P. N. Campbell. (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W.* 1)

A part of the ribonucleic acid present in the soluble fraction of disrupted liver cells has been implicated as an intermediate in protein synthesis. This ribonucleic acid has been termed soluble—or S-RNA. In experiments with isolated liver microsomes the transfer of [^{14}C]amino acid bound to S-RNA to uncharacterized proteins was demonstrated (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). The utilization of such amino acids for the synthesis of serum albumin has now been studied.

A preparation of S-RNA–[^{14}C]amino acid complex with the required specific radioactivity was obtained as follows. The pH 5 fraction from rat-liver-cell sap was repeatedly incubated and reprecipitated to remove the amino acids bound to the S-RNA present in this fraction. The product was then incubated with [^{14}C]leucine as described by Hecht, Stephenson & Zamecnik (1959) and the

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[^{14}C]leucine-labelled S-RNA isolated with phenol. The microsome fraction from either normal or regenerating liver was incubated with the labelled S-RNA, in a tris-buffered medium (Rendi & Hultin, 1960) together with ATP, GTP, pH 5 fraction, phosphoenolpyruvate and pyruvate kinase. The incorporation of [^{14}C]leucine into the insoluble particulate protein and into the serum albumin released from the endoplasmic reticulum by ultrasonics (Campbell, Greengard & Kernot, 1960) was determined. Radioactive serum albumin was obtained. The comparative radioactivity of the serum albumin and the insoluble protein was similar to that obtained when microsomes were incubated with a sufficient quantity of free [^{14}C]leucine.

In control experiments microsomes were incubated with a non-radioactive S-RNA–amino acid complex together with free [^{14}C]leucine of the appropriate radioactivity. In the presence of such low amounts of free amino acid the radioactivity of the serum albumin was insignificant.

The results obtained are consistent with the concept that amino acids attached to S-RNA are transferred to specific soluble protein which is synthesized in the liver microsomes.

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(5) Studies on the Synthesis of Serum Albumin by Isolated Ribonucleoprotein Particles from Rat Liver

By Alexandra von der Decken* and P. N. Campbell. (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W.* 1)

Korner (1960) has reported that [^{14}C]amino acids are incorporated into serum albumin when ribonucleoprotein particles (RNP-particles), prepared by deoxycholate treatment of a rat-liver homogenate, are incubated with a source of ATP. The possible synthesis of serum albumin by a different preparation of RNP-particles using the methods previously described for liver microsomes (Campbell, Greengard & Kernot, 1960) is now reported.

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RNP-particles were isolated from the livers of young fasted rats after treatment of the microsome fraction of the tissue homogenate with deoxycholate, lubrol and KCl as described by Rendi & Hultin (1960). The particles were then suspended in tris-buffered medium and incubated for 60 min. at 37° in the presence of ATP, phosphoenolpyruvate, pyruvate kinase, [^{14}C]leucine and cell sap. The latter was the 105 000g supernatant from a homogenate of rat liver. After incubation the particles were separated by centrifuging and subjected to ultrasonic vibrations, frozen and again centrifuged. A small amount of carrier serum albumin was added to the soluble fraction obtained.

The radioactivity of the serum albumin in (*a*) the soluble part of the incubation medium and (*b*) the soluble part of the ultrasonic extract was determined. In each case the extracts were 'cleared' with either ovalbumin–anti-ovalbumin or duck-serum albumin–anti-duck serum albumin before the addition of an antiserum to rat-serum albumin (Campbell *et al.* 1960).

Other experiments showed that a very small amount of serum albumin was associated with the RNP-particles. This albumin could be released either by incubation at 37° or by ultrasonics.

The results of the radioactivity determinations indicated that there was an energy-dependent incorporation of [^{14}C]leucine into the serum albumin of both fractions (*a*) and (*b*). The most striking results were obtained, however, with extract (*b*). Estimations on the rat-albumin precipitates indicated the presence of some ribonucleic acid in the precipitates from (*b*). When, however, the nucleic acid was removed with hot trichloroacetic acid, the specific radioactivity of the protein was unaffected.

The results obtained are consistent with the concept that isolated rat-liver-ribonucleoprotein particles are able to synthesize rat-serum albumin.

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(6) Studies on the Intracellular Location of Deoxyribonucleic acid Polymerase

By R. M. S. Smellie and R. Eason. (*Department of Biochemistry, The University of Glasgow*)

The intracellular location of the polymerase responsible for the biosynthesis of DNA (Smellie, Keir & Davidson, 1959; Smellie *et al.* 1960) has

been investigated in Ehrlich and Landschutz ascites tumour cells and in rabbit tissues.

Preliminary experiments showed that the incorporation of [^{3}H]thymidine (TdR) into DNA by soluble cytoplasmic extracts of ascites cells was not enhanced but actually depressed by the addition of disrupted ascites cell nuclei to the preparation.

When nuclei were isolated from ascites cells and rabbit tissues in 0·25M-sucrose or 0·9% NaCl and subsequently disrupted, they showed negligible activity with respect to the incorporation of thymidine 5′-[^{32}P]triphosphate (TTP) into DNA in the presence of the 5′-triphosphates of deoxyadenosine, deoxyguanosine and deoxycytidine (dATP, dGTP and dCTP), whereas the corresponding soluble cytoplasmic fractions were highly active.

The failure to detect the DNA polymerase in isolated nuclei could be due to extraction of the enzyme from the nuclei during the course of isolation and experiments were therefore carried out to measure the polymerase activity of nuclei isolated in non-aqueous solvents (Kay, Smellie, Humphrey & Davidson, 1956). The specific activities of DNA polymerase, adenosine deaminase and adenosine triphosphatase from rabbit thymus nuclei were determined (*a*) after the tissue had been lyophylized and disrupted in petroleum ether, (*b*) at an intermediate stage in the isolation of the nuclei, and (*c*) when the nuclei had been obtained free from appreciable cytoplasmic contamination. While the specific activity of the polymerase decreased throughout the purification procedure from about 2000 to 100, the specific activities of the adenosine deaminase and adenosine triphosphatase remained essentially constant. The observations on the latter two enzymes show that there is little non-specific denaturation of enzymes during the isolation procedure and the demonstration of polymerase activity in the crude disrupted preparation shows that the enzyme can withstand treatment with organic solvents. The decline in specific activity of the polymerase as the nuclei are purified must be due to its absence from the nuclei, to its destruction during the isolation procedure or to some other factor as yet unknown, but these results taken in conjunction with those from nuclei isolated in aqueous media tend to suggest that the enzyme is not of nuclear origin.

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(7) Acetyl Groups as *N*-Terminal Substituents in Calf-Thymus Histones

By D. M. P. Phillips. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

The histones of calf thymus have chiefly proline and alanine as *N*-terminal amino acids, amounting to 1 mole per 25 000–35 000 weight of protein (Phillips, 1958). The proline group occurs mainly in the slightly lysine-rich histones (lysine/arginine molar ratio = 1–4) and the alanine group in the arginine-rich histones (lysine/arginine < 1), (Phillips & Johns, 1959). However, the very lysine-rich histones (lysine/arginine > 4) and the main component (F2/*a*) of the slightly lysine-rich group gave only 1 mole of *N*-terminal amino acids from 40 000–270 000 weight of the protein. These weights are far in excess of the molecular weights indicated by physical measurements (cf. Trautman & Crampton, 1959).

The *N*-terminal amino acids are apparently largely masked in these fractions and more vigorous conditions of coupling with fluorodinitrobenzene did not increase them. Other experiments have shown that the *N*-terminal positions are not masked by nucleotide, pyrrolidone carboxylic acid or sarcosine.

On the other hand, acetate was found in acid hydrolysates of these histones, being detected by chromatography as acetate (Brown & Hall, 1950), acethydrazide (Satake & Seki, 1950) and dinitrophenyl acethydrazide, the yield being very approximately 1 mole per 13 000–44 000 weight, depending on the fraction examined. With unfractionated histones the quantity was 1 mole in 60 000–70 000 weight. Acetate was still obtained from lipid-free histone and was present only in traces in the ether extracts of acid solutions of the unhydrolysed proteins, so that it was not derived from uncombined acetate. It is suggested tentatively that the acetyl groups are *N*-terminal substituents in these two histone fractions, which together constitute about half the histones of calf thymus.

The arginine-rich group of histones, which have alanine as the *N*-terminal amino acid amounting to 1 mole in 18 000 weight of the protein (Johns, Phillips, Simson & Butler, 1960) were also found to give acetate amounting to about 1 mole in 30 000 weight. Starch-gel electrophoresis (Johns, Phillips, Simson & Butler, 1961) has shown that this histone group consists of two major components, but this fraction also contained some carbohydrate equivalent to 1 mole of glucose in 53 000 weight, so that in this case the acetyl group may be present in extraneous materials.

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(8) The Presence of Physically Associated Non-Protein Nitrogen in Collagenous Proteins

By F. S. Steven, G. R. Tristram and I. R. Tyson. (*Department of Physiology and Biochemistry, St Salvator's College, University of St Andrews*)

Bowes, Elliott & Moss (1957) detected no *N*-terminal residues in ox-hide collagen and trace amounts of *N*-terminal aspartic acid and alanine in citrate-soluble collagen. In the present work minced dehaired calf skin was suspended in 0·2M-Na_2HPO_4 and disintegrated in a modified hammer mill. The suspension was exhaustively extracted with 0·2M-Na_2HPO_4 to remove non-collagenous proteins before extracting the collagen with 0·1M-acetic acid. The acetic acid-soluble collagen was purified by selective precipitation (five times) by dropwise addition of 30% (w/v) NaCl to the acetic acid solution to give a final concentration of 7% (w/v) NaCl. The insoluble collagen was obtained as a residue from calf skin exhaustively extracted with citrate (Bowes, Elliott & Moss, 1955). The gelatin was a commercial lime-processed hide gelatin.

The original proteins were dissolved in 0·1M-acetic acid and dialysed against this same solution. The dialysable materials were concentrated by vacuum distillation below 60°, desalted on Dowex-50 or Zeocarb 225 columns and the non-protein nitrogen (NPN) eluted with distilled N-NH_3. The non-diffusible materials were precipitated with six volumes of acetone and the acetone-soluble peptides isolated as above. Total nitrogen analyses indicated that the combined NPN fractions accounted for 2% of the original soluble collagen nitrogen. Two-dimensional paper chromatography demonstrated the presence of eleven NPN components which were common to both the diffusible and acetone-soluble fractions obtained from each of the three original proteins. The acetone-soluble NPN was shown to be free to dialyse in the absence of collagen. The *N*-terminal residues of the original proteins, diffusible and acetone-soluble fractions were demonstrated to be identical in all cases; these were Ala, Asp, Glu, Gly, Leu and/or Ileu, Lys, Phe. Ser, Thr, and Val. The acetone-insoluble residues from the acetic acid-soluble collagen and gelatin had the same pattern of *N*-terminal residues with the exception of Leu and/or Ileu. The preparation of an identical pattern of eleven NPN components from three widely different collagenous proteins suggests that the NPN has an important function in the intact collagen complex of skin. The presence of low-molecular-weight materials in gelatin solutions may explain the difference between the physical molecular weight (300 000) and average chemical molecular weight (60 000) obtained by end-group studies (Courts & Stainsby, 1958).

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(9) The Interaction of Phosvitin with Cations

By W. M. McIndoe. (*Agricultural Research Council, Poultry Research Centre, West Mains Road, Edinburgh* 9)

The polyphosphorylated protein phosvitin was prepared by the following procedure. Hen-egg yolk was mixed with two volumes of 0·1M-NaCl and centrifuged for 1 hr. at 20 900*g*. The particulate material was washed twice with 0·1M-NaCl, dissolved in 0·1M-trisodium citrate, and dialysed against the same solvent. The solution was mixed with 0·5 volumes of butanol to remove lipid from the 'lipovitellins', rendering the protein moiety insoluble but leaving the phosvitin in solution in the clear, colourless aqueous phase (Morton, 1950; Sundararajan, Sampath Kumar & Sarma, 1960). This preparation had an N/P ratio of 2·75, within the range of published values.

Other methods of preparation (Mecham & Olcott, 1949; Joubert & Cook, 1958) have used Mg^{2+} to precipitate phosvitin. However, Ca^{2+} precipitates phosvitin at lower concentrations than will Mg^{2+} and cetyl pyridinium chloride causes precipitation when two molecules are present for every atom of phosvitin P. Conditions for the precipitation of phosvitin by the above cations were presented in detail and consideration given to the possibility of using them in its isolation without the use of butanol.

Phosvitin also reacts in various ways with basic dyes. For example it precipitates on the addition

of acridine orange: toluidine blue exhibits metachromasia in the presence of phosvitin but no precipitation occurs. Further experiments with these and other dyes were described.

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(10) Determination of Mannose in a Protein by a Radioisotope Dilution Method

By C. François, R. D. Marshall and A. Neuberger. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2*)

Colorimetric techniques involving the use of phenols and concentrated sulphuric acid have often been employed for the estimation of sugars in glycoproteins and polysaccharides. However, it is not known whether a solution containing a given amount of a sugar will give a colour yield identical with that obtained from the same amount of sugar but combined in polysaccharide linkage (cf. Vasseur, 1948). For this reason we have compared the values for the mannose content of hen's egg albumin (2·0%) (Johansen, Marshall & Neuberger, 1960) obtained by an orcinol–sulphuric acid procedure (Winzler, 1955), with those obtained by a radioisotope dilution method.

Egg albumin (usually about 2 g.) was hydrolysed in 2N-HCl at 100% after the addition of a known quantity of D-[^{14}C]mannose. When hydrolysis had occurred for a measured period of time, the mannose in solution was isolated as the phenylhydrazone and the amount of mannose liberated was calculated. This was found to be equivalent to 2·00% after 2 hr., 2·07% after 3 hr., 2·06% after 4 hr., and 2·01% after 5 hr. hydrolysis, based on the ash-free, moisture-free protein. Because of the acid lability of free mannose these values are likely to be a measure of the maximum amount of mannose that is liberated by hydrolysis under these conditions. There is, however, the possibility that there is still a greater amount of mannose in the glycoprotein than we have observed, and indeed can observe, if the mannose were combined with C-1 of glucosamine (Gottschalk & Ada, 1956; Johansen *et al.* 1960).

Hydrolysis of the protein without added [^{14}C]-mannose in 2N-HCl at 100° was also carried out. The mannose was isolated after various periods of time as the phenylhydrazone and gave values of mannose equivalent to 1·55% (2 hr.), 1·77% (3 hr.), 1·40% (4¼ hr.), and 0·68% (6 hr.) of the protein.

Egg albumin must therefore contain at least 1·77% of mannose (4·5 residues per 45 000 g. of protein) and probably not more than 2·0% (5·0 residues), the latter value being not absolutely certain for the reasons previously discussed (Johansen *et al.* 1960).

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(11) Enzymic Degradation of Lichenin

By W. L. Cunningham and D. J. Manners. (*Department of Chemistry, University of Edinburgh*)

Lichenin, a reserve polysaccharide from Iceland moss, was shown by methylation and periodate oxidation studies to be a linear polymer composed of 70% β-1:4- and 30% β-1:3-linked D-glucopyranose residues (Chanda, Hirst & Manners, 1957). Partial acid hydrolysis studies (Peat, Whelan & Roberts, 1957) then showed that the major repeating sequence consisted of β-cellotriose units joined through 1:3-linkages. We now report the degradation of this polysaccharide by two different laminarinase preparations.

Extracts of malted barley, like those of ungerminated barley (Manners, 1955), contain several carbohydrases, including cellobiase, laminaribiase, cellodextrinase and laminarinase. In the presence of 10 mM-glucono-1:4-lactone (cf. Festenstein, 1959) the latter activity is reduced by 67% but the other activities are completely inhibited. Under these conditions, slow random hydrolysis of lichenin occurred (paper chromatography), the major product having the R_G value of a trisaccharide; small amounts of glucose, laminaribiose and a sugar with the R_G value of a tetrasaccharide were also produced.

The same oligosaccharides were formed when lichenin was incubated with an enzyme preparation from the culture medium of *Rhizopus arrhizus*, known to produce a laminarinase (Reese & Mandels, 1959).

The latter trisaccharide has been isolated by chromatography on Whatman no. 3MM paper and characterized as 3-*O*-β-cellobiosyl-D-glucose; paper chromatographic analysis showed that (α) partial acid hydrolysis gave glucose, laminaribiose and

cellobiose, (*b*) after hypoiodite oxidation, partial acid hydrolysis gave glucose, gluconic acid and cellobiose and (*c*) hydrolysis with emulsin gave glucose and laminaribiose. The specific rotation ($[\alpha]_D + 12{\cdot}3°$ in water) is in good agreement with that ($[\alpha]_D + 12{\cdot}7°$) reported by Peat *et al.* (1957) and by Parrish, Perlin & Reese (1960) who isolated the same trisaccharide ($[\alpha]_D + 12{\cdot}1°$) from a digest of *Rhizopus arrhizus* laminarinase and oat glucan (a polysaccharide closely related to lichenin in molecular structure).

Since lichenin does not contain a sequence of two adjacent 1:3-linkages (Chanda *et al.* 1957; Peat *et al.* 1957) enzyme action must involve the rupture of a β-1:4-glucosidic linkage. The specificity of laminarinase may therefore be defined by

$$-\mathrm{G}\overset{(a)}{-}\mathrm{G}\overset{(b)}{-}\mathrm{G}-$$

where G represents a β-D-glucopyranose residue, (*a*) a 1:3-linkage and (*b*) the linkage hydrolysed, which may be a 1:3- or a 1:4-linkage. These conclusions are in exact agreement with those of Parrish *et al.* (1960).

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(12) Hydrolysis of Ester Sulphate from Seaweed Polysaccharides

By J. P. McKinnell and Elizabeth Percival.
(*Department of Chemistry, University of Edinburgh*)

The water-soluble polysaccharide of the green seaweed, *Enteromorpha compressa*, after separation of a starch-type glucan, has $[\alpha]_D - 87°$ and consists of at least 40% of rhamnose units together with xylose, glucose, glucuronic acid (18%) and 16% of ester sulphate. Infrared analysis of this polysaccharide reveals a peak at 850 cm.$^{-1}$ indicating the presence of axial sulphate (Lloyd, Dodgson, Price & Rose, 1961). The ester sulphate groups are stable to alkali and preferentially to remove them with mineral acid without cleavage of the glycosidic links was not possible.

Kantor & Schubert (1957) reported the removal of sulphate groups from chrondroitin by the action of 0·06M-methanolic hydrogen chloride, but application of this method to fucoidin, the sulphated polysaccharide from brown seaweeds, was unsuccessful. Extensive hydrolysis of the glycosidic links occurred and the polysaccharide, recovered in low yield, contained a slightly higher proportion of sulphate than the original fucodin. However, on shaking the sulphated polysaccharide from *E. compressa* with 0·09M-methanolic hydrogen chloride at room temperature for 24 hr. a 70% yield of polysaccharide material was recovered (after dialysis) in which the sulphate content had been reduced to 1%. Analysis of a hydrolysate of this material revealed the presence of the same monosaccharides, in approximately the same proportions, as the original polysaccharide.

The original material reduced 0·38 mole of periodate (Cantley, Hough & Pittet, 1959; Hough & Perry, 1956) for every C_6 sugar residue, whereas the desulphated material reduced 0·68 mole for the same weight of polymer. This low reduction of periodate indicates that the polysaccharide contains a high proportion of 1:3-linked residues or that it is highly branched. The higher consumption of periodate by the desulphated material reveals that the removal of sulphate produced additional units with adjacent free hydroxyl groups.

Estimation (Wilson, 1959; Dische & Shettles, 1948) of the relative proportions of the monosaccharides in the two periodate oxidized polysaccharides indicated no obvious change in the relative proportions of the sugars except that the desulphated polysaccharide had a lower proportion of rhamnose units, thus showing that the sulphate groups are mainly attached to rhamnose. In the Cl conformation of rhamnose only $C_{(2)}$ carries an axial hydroxyl group and it appears probable therefore that it is this carbon atom that carries the sulphate. This is the first direct evidence of the site of the sulphate group in sulphated polysaccharides of the green algae.

We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest and encouragement and the Department of Industrial Research for a Maintenance Allowance (to J.P.M.).

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(13) Identity of β-Glucosidase and β-Galactosidase in Sweet-Almond Emulsin

By R. Heyworth and P. G. Walker. (*Biochemistry Department, Institute of Orthopaedics, Brockley Hill, Stanmore, Middlesex*)

Helferich has reported considerable evidence that the β-glucosidase and β-galactosidase activities of sweet almond meal were associated with the same enzyme protein (see Veibel, 1950). However Veibel, Wangel & Østrup (1947) were unable to demonstrate an inhibition of β-glucosidase by galactose and found a difference in K_i for glucose when measured against the two *o*-cresyl glycosides. They concluded that the two hexosidases were different.

p-Nitrophenyl galactoside has been prepared and the enzymic liberation of *p*-nitrophenol from this substrate and from *p*-nitrophenyl glucoside, at concentrations of 1–8 mM, has been measured according to Woollen, Heyworth & Walker (1961). These activities (in 0·05M-butyrate buffer) were proportional to the concentration of the buffered meal extract and to time (20 min.). The pH optimum for β-glucosidase was 5·5 and for β-galactosidase, 5·6.

Ammonium sulphate fractionation of the enzyme and partial heat inactivation (at 65°) to 27 % of the original activities gave no change in the ratio of the two activities (*s*, 8 mM; pH 5·6).

The K_m values were 2·6 mM for *p*-nitrophenyl glucoside and 13 mM for *p*-nitrophenyl galactoside at pH 5·6. The liberation of *p*-nitrophenol in the presence of equimolar mixtures of the two substrates was that predicted on the assumption of hydrolysis of both substrates at one enzyme site.

Glucose is a competitive inhibitor of both activities with K_i values of 0·21M when measured against the glucoside and 0·26M when measured against the galactoside. Galactose is similarly a competitive inhibitor with corresponding K_i values of 0·64M and 0·56M.

It is concluded that the hydrolysis of both substrates takes place at the same enzyme site. Similar lack of specificity at C-4 has been demonstrated for β-glucuronidase (Marsh & Levvy, 1958) and for *N*-acetyl-β-D-glucosaminidase (Woollen *et al.* 1961).

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(14) Plasma Acid Phosphatase Activity and Bone Dystrophies in the Domestic Fowl

By D. J. Bell. (*Agricultural Research Council, Poultry Research Centre, Edinburgh* 9)

A small plasma acid phosphatase activity has recently been reported in fowls up to 6 months of age (McDaniel & Chute, 1961). By adapting the method used for alkaline phosphatase (Bell, 1960), acid phosphatase activity against 4-nitrophenyl phosphate has been studied in normal birds of different ages and in cases involving osteodystrophies. Units used below are mg. of 4-nitrophenol freed by 100 ml. of plasma in 1 hr. in 0·2M-citrate or acetate buffers of pH 4·9 at 37°.

The following mean values have been obtained for different age groups of normal birds (figures in parentheses indicate numbers examined): 5 weeks (10), 5·00, S.D. $\pm$ 2·81; 7 weeks (11), 3·36 $\pm$ 0·81; 15 weeks (14), 6·50 $\pm$ 1·95; 18 weeks (7), 7·43 $\pm$ 1·72; 9- to 12-month-laying pullets (13), 5·85 $\pm$ 2·81. Older adults of both sexes resemble pullets; in individual females levels of acid phosphatase activity remain steady during several months of laying.

Fowls with severe osteoporosis, where many osteoclasts are present, usually show an elevated plasma acid phosphatase activity (as well as greatly raised alkaline phosphatase activity). In nine pullets with 'cage-layer fatigue' (Bell, Siller & Campbell, 1959) where excessive bone resorption is seen, plasma acid phosphatase activities were 12, 14, 19, 20, 21, 22, 25, 28 and 30.

Laying pullets transferred to a diet containing but 1 % of calcium became severely osteoporotic (cf. Taylor & Moore, 1955) and frequently collapsed in a condition simulating, but not identical with 'cage-layer fatigue'. Five cases showed plasma acid phosphatase activities of 47, 71, 31, 22 and 28.

Osteoclasts contain acid phosphatase (Changus, 1957; Burstone, 1959; Peplar, 1960). It seems therefore possible that the extra acid phosphatase activity of the plasma of osteoporotic birds stems from an increased turnover of osteoclasts.

No similar observation is known to the author with the exception of a raised plasma acid phosphatase activity reported in a human case of parathyroid adenoma (Klendshoj & Koepf, 1943). Fowls with florid osteopetrosis, where osteoclasts are rare, do not show differences from normal plasma levels in this enzyme (Bell & Campbell, 1961).

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(15) Isolation of a Phosphatidylglycerol and a Glycolipid from *Micrococcus lysodeikticus* Cells

By M. G. Macfarlane. (*Lister Institute of Preventive Medicine, London, S.W.* 1)

The phospholipid fraction from the protoplast membrane of *Micrococcus lysodeikticus* consisted of a diphosphatidylglycerol (G-P-G-P-G-lipid) with mainly C_{15} branched acids, some phosphatidylinositol, and a complex yielding glycerophosphate, glycerol, mannose and fatty acids on acid hydrolysis (Macfarlane, 1961). Examination of the lipid from whole cells of *M. lysodeikticus* indicates that this complex was a mixture of a phosphatidylglycerol (G-P-G-lipid) and a glycolipid yielding a glycerylmannoside.

Methods of extraction and analysis, etc., were those used by Macfarlane & Gray (1958). R_F values refer to chromatography on silicic acid paper in di*iso*butylketone–acetic acid–water (40:20:3, by vol.) at 2°. The lipid from 23 g. dry wt. of cells (18 mg. of P, 57 mg. of carbohydrate) was fractionated on silicic acid. Only 4% of the P was eluted as G-P-G-P-G-lipid; this was followed by the glycolipid (fraction II, 39 mg. of carbohydrate) with molar ratio fatty acid ester: carbohydrate: P, 5·7:3·2:1·0, and then by the overlapping G-P-G-lipid (fraction III) containing 72% of the total P (molar ratio fatty acid ester:P:carbohydrate, 1·9:1·0:0·1). The remaining P (12·9%) was eluted as virtually pure phosphatidylinositol, containing predominantly C_{15} branched acids. Some carbohydrate was not recovered.

Fraction II, purified by extraction into acetone, was soluble also in ether, chloroform and ethanol; $[\alpha]_D$ +35° in chloroform (*c*, 0·23); reducing sugar after acid hydrolysis 26%; fatty acids recovered on saponification, 56%; R_F about 0·5. The water-soluble saponification product was non-reducing, but on acid hydrolysis gave glycerol and mannose, identified by paper chromatography, in equimolar proportion, as estimated by differential periodate oxidation. On oxidation of the intact lipid at room temperature with periodic acid in chloroform–95% acetic acid (1:1, v/v), 2 moles of periodate per mole of sugar were consumed, without production of formaldehyde, indicating that the mannose moiety was not acylated in positions 2, 3 or 4. By colorimetric assay, with a lecithin standard, the ratio fatty acid ester:sugar was 1·5 in the intact lipid, and 1·7 in the recovered saponification products. The fatty acids were mainly the *anteiso* and *iso* C_{15} acids; their position is not known.

Fraction III was purified by the sparing solubility of the Na salt in ether at 2°; the salt was soluble in chloroform and ethanol; P 4·6%; $[\alpha]_D$ +5° in ethanol (*c*, 0·6); molar ratio fatty acid ester:P:carbohydrate, 1·95:1·0:0·02; R_F about 0·5. On oxidation with periodic acid the intact lipid consumed one mole of periodate per mole of P and gave one mole of formaldehyde. The water-soluble ester (G-P-G) obtained on saponification gave 2·1 moles of formaldehyde per mole of P with sodium periodate at room temperature; it was distinguished from glycerophosphate (GP) and G-P-G-P-G by paper chromatography in phenol–water (100:38, v/v) and butanol–acetic acid–water (5:3:1, by vol.) (cf. Benson & Maruo, 1958; Kates, 1960).

The pooled fatty acids from the bacterial lipids, mainly the *anteiso* C_{15} acid, had $[\alpha]_D$ +3° (*c*, 3·5) as methyl esters in chloroform.

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(16) Phospholipids of Isolated Wheat Gluten

By C. B. Coulson and Elspeth A. Somerville. (*Arthur D. Little Research Institute, Inveresk, Midlothian*)

Work on unhydrolysed wheat lipids has, so far, been confined to those of flour (e.g. Fisher & Broughton, 1960; Wren & Elliston, 1961). Both groups describe fractionation on silicic acid columns leading to 10 to 14 major components, but it is a somewhat complicated procedure where the initial interest is the comparison of different lipid extracts. Various reports have been made of separations of phospholipids by paper chromatography (Witter, Marinetti, Heicklin & Cottone, 1958; Lea, Rhodes & Stoll, 1955). The best results were obtained using silicic acid-impregnated paper

(Macfarlane, 1961). The developing solvent was di-*iso*butyl ketone–acetic acid–water (40:20:3, by vol.) and the stain Rhodamine 3 GO (0·001 %, w/v). Due to the impermeability of wheat gluten, extraction of the phospholipids is more difficult than with flour. Maceration in chloroform–methanol (2:1, v/v), or water-saturated butanol, was found to give the best results. Neither acetone nor carbon tetrachloride were successful. Using the above method 13 components have been found in chloroform–methanol and water-saturated butanol extracts. The bands on the chromatograms from the two extracts are not identical. Aqueous NaCl washing of the gluten has been thought to remove much lipid. Preliminary experiments (with 2M-NaCl) would indicate that only lipids having very low R_F values are removed.

A rapid simple method for estimating the amount of phospholipid in a solution has been developed [suggested by the method of Gootjes & Nauta (1954) for glycyrrhizin] for use with column chromatography eluates. A linear relation between the amounts of phospholipid in solution and the intensity of colour obtained, when a standard amount of Rhodamine 3GO is added to the solution, exists over quite a range. Various solvents have been tested, e.g. ethanol, methanol, methanol–chloroform (1:1, v/v), acetic acid (aqueous 90 %) and water-saturated butanol. Where the solvent used was immiscible with water, a chloroform solution of the dye was used. Phospholipid levels at least as low as 0·25 mg./ml. (approx. 10 μg. of P) can be detected. The addition of ammonia to the coloured solution did not significantly increase the colour intensity although the spot colour on the chromatogram is enhanced by ammonia fumes.

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(17) Starch-Gel Electrophoresis of Isolated Wheat Gluten

By C. B. Coulson and A. K. Sim. (*Arthur D. Little Research Institute, Inveresk, Midlothian*)

Isolated wheat gluten was shown by Elton & Ewart (1960) to consist of at least eight components when fractionated by starch-gel electrophoresis [standard gel but aluminium lactate buffer, Jones, Taylor & Senti (1959), Naphthalene Black Stain]. We find that by concentrating with Carbowax 20M (G. T. Gurr Ltd.), reducing the concentration of starch in the gel (to 10 %, Connaught Starch-Hydrolysed; 8-10 v/cm. for approximately 2 hr.) and cutting extensive colour loss by staining in Nigrosine (Smithies, 1959), more than twenty components can be observed.

After washing the flour in butanol and pyrophosphate solution, Simmonds & Winzor (1961) fractionated the acetic acid extract on a carboxymethylcellulose column into six components. The presence of an additional two components was attributed to incomplete removal of aqueous-salt-soluble 'non-gluten' proteins (Simmonds & Winzor, 1961; cf. Pence, Mecham & Olcott, 1956). Proteins were isolated from wheat flour by the Simmonds & Winzor (1961) procedure. On examination by starch-gel electrophoresis we find the 2M-sodium pyrophosphate solution (pH 7) only partly extracts several components, mainly of an intermediate mobility. On standing at 4° for several days, changes occurred in the concentrations of the gluten fractions dispersed in 0·1N-acetic acid, although the overall qualitative pattern remained constant. The faster-moving components became more concentrated at the expense of those of lower mobility. This may be due to protein dissociation, perhaps partly into the genetic protein units, with the establishment of a new equilibrium, in which case the pyrophosphate-soluble 'non-gluten' components may include some of the basic components of gluten. Ethanol-soluble gliadin (British Drug Houses Ltd) was found to correspond to the slower-moving fractions—in agreement with the free boundary electrophoresis findings of Jones *et al.* (1959).

The gluten components, present in acetic acid solutions, do not appear to succumb readily to denaturing agents, unlike most proteins. Short heating times (95°; 3 min.) do not appear to alter their solubility or their mobility in starch gel. The addition of urea (to about 8M) to the protein solution before electrophoresis does not cause any change in the patterns observed. Some proteins are known to regain their native state when the urea-containing solution is diluted (Kauzmann, 1959).

The changes in gluten constituents on standing suggest that even greater changes take place when gluten is dispersed. Yet gluten in solution is relatively difficult to denature by heating and probably only suffers reversible denaturing in urea.

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(18) The Biosynthesis of Fatty Acids in Extracts of Lactating Rat Mammary Glands

By R. Dils and G. Popják. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London, W.* 12)

Previous work on lactating mammary gland suggested that fatty acid biosynthesis from acetate takes place by reactions analogous to the reversal of β-oxidation (Hele, Popják & Lauryssens, 1957). More recent work with avian liver has shown that fatty acid synthesis involves the carboxylation of acetyl–CoA to malonyl–CoA and subsequent condensation of malonyl–CoA with acetyl–CoA (Brady, 1958; Wakil & Ganguly, 1959). The synthesis of fatty acids by lactating rat mammary glands has been re-investigated to determine whether this malonyl–CoA pathway is also applicable to this tissue.

Homogenates were prepared by the method of Popják & Tietz (1954) and fractionated by high-speed centrifugation (Popják & Tietz, 1955). Optimum synthesis of fatty acids from acetate occurred in the particle-free supernatant when the cofactors required for synthesis from acetate by the avian liver system (i.e. ATP, CoA, TPNH, HCO_3^- and Mn^{2+}) were present. A slight requirement for DPNH was observed. Malonate strongly stimulated synthesis, as had been first observed by Popják & Tietz (1955) and subsequently seen in rat-liver systems (Abraham, Matthes & Chaikoff, 1960; Fletcher & Myant, 1961).

Since [1-^{14}C]- and [2-^{14}C]-malonic acid were not incorporated into the synthesized fatty acids, the rat mammary gland fatty acid synthesizing system lacks malonic kinase, as does the rat liver system (Abraham *et al.* 1960). One explanation for the stimulation by malonate might be that it prevents the deacylation of malonyl–CoA formed from acetate. The addition of increasing concentrations of avidin (which inhibits the biotin-dependent carboxylation of acetyl–CoA to malonyl–CoA) in the presence of optimum amounts of malonate and HCO_3^-, resulted in an almost total inhibition of synthesis of fatty acids from acetate. This showed that under conditions for optimum synthesis via malonyl–CoA, virtually no synthesis of fatty acids by the reversal of β-oxidation occurred. Since these results were obtained, Abraham, Matthes & Chaikoff (1961) have shown that avidin also suppressed fatty acid synthesis from acetate in the mammary gland in the absence of added malonate.

When particle-free extracts of mammary gland were supplemented with all the necessary cofactors and malonate, members of the citric acid cycle which were previously reported to stimulate synthesis of fatty acids (Popják & Tietz, 1955) were without effect. Oxaloacetate (12·5 mM) still stimulated synthesis under these conditions, however.

The main products of synthesis were all the even-numbered saturated fatty acids from C_6 to C_{16}; details of their composition were given.

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(19) Phosphomevalonic Kinase and 5-Pyrophosphomevalonic Anhydrodecarboxylase from Pig Liver

By Hermione Hellig and G. Popják. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London, W.* 12)

Phosphomevalonic kinase (Bloch, Chaykin, Phillips & de Waard, 1959; Henning, Möslein & Lynen, 1959; Levy & Popják, 1960) and 5-pyrophosphomevalonic anhydrodecarboxylase (Bloch *et al.* 1959) catalyse two of the steps in the formation of *iso*pentenyl pyrophosphate (IPP) from mevalonic acid. These enzymes have been isolated from pig liver, partially purified and their properties studied. As no satisfactory chemical synthesis exists for the preparation of the substrates for these enzymes both (−)-5-phosphomevalonic acid (Cornforth, Fletcher, Hellig & Popják, 1960) and (−)-5-pyrophosphomevalonic acid were made from DL-[1-^{14}C]- and DL-[2-^{14}C]-mevalonic acid with purified liver enzymes (Levy & Popják, 1960).

Phosphomevalonic kinase catalyses the forma-

tion of 5-pyrophosphomevalonate (5-PP-MVA) from 5-phosphomevalonate (5-P-MVA) and ATP:

$$5\text{-P-MVA} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} 5\text{-PP-MVA} + \text{ADP}.$$

In the presence of excess ATP the forward reaction is favoured and can be driven to completion by the regeneration of ATP with a coupled system (e.g. with pyruvic kinase and phosphoenol pyruvate). With an excess of ADP the reaction can be reversed. The enzyme was purified about 20-fold; the best preparations, which were free from other enzymes acting on mevalonic acid or its derivatives, converted, per mg. of protein, 0·078 μmole of 5-P-MVA into 5-PP-MVA per min. at 37°. The optimum conditions for this enzyme were: pH 7·3, 5 mM-$MgCl_2$, 3·6 mM-ATP and mM-5-P-MVA. The K_m for the enzyme is about 0·3 mM. 5-PP-MVA is laevorotatory, as is 5-P-MVA.

The reaction catalysed by 5-pyrophosphomevalonic anhydrodecarboxylase (cf. Popják & Cornforth, 1960):

$$5\text{-PP-MVA} + \text{ATP} \xrightarrow{Mn^{2+}(Mg^{2+})} \text{IPP} + CO_2 + \text{ADP} + P_i,$$

proceeds at the maximum rate at pH 5·1 in the presence of 2 mM-ATP, 5 mM-$MnCl_2$ and 0·1 mM-5-PP-MVA. The K_m for the enzyme is about 0·5 μM. The best preparations decarboxylated about 0·014 μmole of 5-PP-MVA per min./mg. protein at 37°.

As the affinity of phosphomevalonic kinase for its substrate is much lower (K_m = 0·3 mM) than of mevalonic kinase for mevalonate (K_m = 0·05 mM), the reversible phosphomevalonic kinase reaction might have been rate-limiting in the conversion of mevalonate into squalene. However, the high affinity of the decarboxylase for 5-PP-MVA would assure in the cell the completion of the phosphomevalonic kinase reaction.

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(20) The Biosynthesis of Carotenoids in *Rhizophlyctis rosea*

By B. H. Davies. (*Department of Agricultural Biochemistry, University College of Wales, Aberystwyth*)

The chytridiaceous phycomycete, *Rhizophlyctis rosea* (de Bary & Woronin) Fischer, when cultured on cellulose lens tissue or cellobiose in a mineral salt solution, becomes orange-pink in three weeks. The pigmentation is due to several carotenoids, lycopene and γ-carotene being predominant, each constituting about 45% of the total carotenoid fraction (Davies, 1961*a*). The synthesis of the carotenoids is independent of illumination. The orange-pink colour is characteristic of mature (3-week) cultures; young (7- to 10-day) cultures are rose-pink. Examination of the pigment extract from young cultures by absorption chromatography on kieselguhr-impregnated paper, with light petroleum as the developing solvent (Jensen & Jensen, 1959; Davies, 1961*b*), revealed that lycopene was the only carotenoid present. γ-Carotene only appears in cultures more than 10 days old, when lycopene synthesis is virtually complete.

R. rosea was cultured on a cellobiose–mineral salts medium for 10 days, and then washed and resuspended in phosphate buffer, pH 7·0. On reincubation, no γ-carotene synthesis occurred and the culture remained pink, even on addition of cellobiose or glucose to the buffer. This suggested that γ-carotene is not synthesized from lycopene by ring-closure, but that it has an independent biosynthetic pathway. Further evidence for the existence of separate pathways was provided by adding [2-^{14}C]mevalonate to cultures of *R. rosea* at different stages of development, isolating the two carotenoids from the mature cultures and comparing their specific activities. When [2-^{14}C]-mevalonate was added at inoculation and was present throughout the incubation period, the specific activities of the two carotenes were approximately equal. If the addition of the labelled precursor was delayed until 10 days after inoculation, then the specific activity of the γ-carotene was at least 10 times that of lycopene. On the other hand, the addition of [2-^{14}C]mevalonate to a culture at inoculation, and its removal after 10 days' incubation, resulted in lycopene having a specific activity 7 times greater than that of γ-carotene.

Cultures of *R. rosea* were kindly provided by Dr E. Griffiths and Mr D. Jones, Department of Agricultural Botany, U.C.W., Aberystwyth, and the [2-^{14}C]mevalonate by F. Hoffman-La Roche and Co. Ltd., Basle. I am grateful to Professor T. W. Goodwin for his interest and advice, and to the Agricultural Research Council for a Research Studentship.

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(21) Δ^4-Steroid Hydrogenase in Livers of Rats Bearing the Walker 256 Carcinoma

By G. A. J. GOODLAD and CATHERINE M. CLARK. (*Department of Biochemistry, St Salvator's College, University of St Andrews*)

Adrenal hypertrophy is a common feature of experimental tumour-bearing animals (Begg, 1958). Urquhart, Yates & Herbst (1959) found a close correlation between adrenal size and the capacity of the liver to inactivate adrenal cortical hormones by ring-A reduction under various physiological and experimental conditions. These workers have further suggested that it is the level of Δ^4-steroid hydrogenase in the liver which determines adrenal size rather than the reverse.

In a study of changes in the tissues of the host during the early stages of growth of the Walker 256 carcinoma in rats it was observed that there was an increase in the total hepatic Δ^4-steroid hydrogenase 3 days after implantation of the tumour. Assay of Δ^4-steroid hydrogenase activity was carried out on liver slices using corticosterone as substrate essentially as described by Yates, Urquhart & Herbst (1958). This rise in Δ^4-steroid hydrogenase activity was accompanied by an increase in the weight of the adrenals. The adrenals continued to hypertrophy during the subsequent stages of tumour growth while the hepatic Δ^4-steroid hydrogenase activity remained at almost twice the normal level during the period studied.

Adrenal hypertrophy may therefore arise in tumour-bearing rats as a result of an increase in the capacity of the livers of such animals to inactivate adrenal corticoids. Thus tumour-bearing animals may have hypertrophied adrenals without being in a hypercortical condition. This view is supported by reports of normal eosinophil patterns in tumour-bearing mice (Halberg, Vermund & Bittner, 1956) and also by some studies on the level of hepatic L-glutamate–pyruvate transaminase which were carried out concurrently with the present work. This enzyme has been shown to increase in concentration in the hypercortical animal (Rosen, Roberts, Budnick & Nichol, 1959). It was found, however, that L-glutamate–pyruvate transaminase in the livers of rats bearing the Walker 256 carcinoma fell to almost 50% of the normal value although the adrenals were in an hypertrophied condition.

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(22) A Method for the Identification of Catalase in Starch Gels

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During an investigation of the enzymes of human cells one of us required a technique which identified catalase. The methods which have been used for detection of this enzyme are those utilizing the production of acetaldehyde from monoethyl hydrogen peroxide (Nishiyama & Kobayashi, 1953) and of bubbles in gel saturated with hydrogen peroxide (Uriel, 1958). Difficulties in utilizing these methods for our purpose led to the development of an alternative technique which we wish to report here. It is based on a standard iodine titrimetric procedure for catalase (Stern, 1932).

The enzyme is isolated from the supernatant of homogenates of cells by electrophoresis in starch gels using a discontinuous buffer system (Poulik, 1957). Slices of gel are cooled to 0–4°, care being taken not to touch the gel with bare fingers as this will lead to patchy development of the background colour. The gel is covered with, and floated free, in 0·01 M-hydrogen peroxide. It is left in this solution for 2 hr. at 0–4°. After decanting the hydrogen peroxide the gel is flooded with cold 2% potassium iodide and kept at 0–4° overnight or until it turns blue or black.

The colour is due to the reaction of free iodine, which is formed from the potassium iodide in the presence of hydrogen peroxide, with the starch gel. Catalase will destroy the peroxide; thus the starch will remain uncoloured where the enzyme is present and a distinct white band will be seen. The band is somewhat slower than 1·1 haptoglobin. The gel can be washed in distilled water and photographed with minimal exposure to light. It may be stored in the dark in water or dilute acetic acid [but not in the usual methanol–acetic acid–water mixture (5:1:5, by vol.)].

In a gel stained in this way, a reddish band, probably attributable to α-amylase, can be seen in runs of serum, not far from the origin. If the gel is left exposed to light this band will persist when the background fades.

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The technique has been used successfully for catalase over the past year by ourselves and others (Paul & Fottrell, 1961).

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(23) Histamine Inactivation in Rat-Kidney Slices

By A. A. Aziz (introduced by P. B. Marshall). (*Department of Pharmacology and Therapeutics. Queen's College, Dundee*)

Sex difference in urinary excretion of histamine in rats has been reported by Marshall (1961), Westling (1958), Kim (1959), Gustafsson, Kahlson & Rosengren (1957) and Leitch, Debley & Haley (1956). The free histamine excretion of female rats is about 10 times that of the males. Histaminase is one of the major enzyme systems which inactivates histamine in rats and aminoguanidine is its specific inhibitor. However, injecting rats with aminoguanidine caused the same proportionate increase in histamine excretion but did not alter the difference between the sexes. Brown, Axelrod & Tomchick (1959) and Brown, Tomchick & Axelrod (1959) demonstrated the presence of histamine methyl transferase in cell-free extracts from different species including the rat, in which the most active tissue is the kidney. The cofactor *S*-adenosyl methionine was used as the active methyl donor.

In the present study, kidney slices were used since with homogenate the activity was negligible. With slices the activity was equivalent to about 45 μg. of histamine inactivated per g. per hour without adding the cofactor *S*-adenosyl methionine. The following properties of this enzymic reaction have been observed:

(1) Comparing four different gas phases: pure O_2, CO_2 (5 %) + O_2 (95 %), pure N_2, and N_2 (95 %) + CO_2 (5 %), the percentage inactivation of histamine was 80, 45, 20 and 18 respectively.

(2) Addition of ATP, methionine, reduced glutathione alone or together was without significant effect on the inactivation of histamine.

(3) The enzyme system contains essential –SH groups since –SH blocking agents such as *p*-chloromercuribenzoate, iodoacetic acid, mercuric chloride and arsenate inhibited the inactivation of histamine. The highest inactivation was with *p*-chloromercuribenzoate and the least active was arsenate.

(4) There is a real sex difference based on body weight in the ability to inactivate histamine, which agrees with the findings *in vivo*. The male-rat kidney has more than double the activity of the female.

(5) Incubation of female-kidney slices with testosterone (10 μM) did not increase the activity.

(6) Preincubation of slices with 5 mM-aminoguanidine was without inhibitory effect on histamine inactivation.

(7) The optimum pH for the reaction was 7·4 and the activity was the same in phosphate buffer as in Krebs–Ringer phosphate buffer.

(8) EDTA (10^{-2}M) had no inhibitory effect, indicating that metallic cofactors such as magnesium or calcium are not essential to the reaction.

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DEMONSTRATION

Horizontal Paper Electrophoresis

By H. F. Pegler and R. Mills (introduced by W. F. J. Cuthbertson). (*Glaxo Laboratories Ltd., Greenford, Middlesex*)

Interlocking polythene bristle mats (14 cm. × 18 cm., Bex Plastic door mats, Halex Ltd, Highams Park, London, E. 4) have been used to make an inexpensive apparatus for horizontal paper electrophoresis with a small air space (about 1 cm.) above the paper, capable of taking papers or strips up to 36 cm. wide and with a range of lengths between 15 and 40 cm.